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(54) Title: NUCLEIC ACID SEQUENCES TO PROTEINS INVOLVED IN TOCOPHEROL SYNTHESIS

(57) Abstract

Nucleic acid sequences and methods are provided for producing plants and seeds having altered tocopherol content and compositions. The methods find particular use in increasing the tocopherol levels in plants, and in providing desirable tocopherol compositions in a host plant cell.

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NUCLEIC ACID SEQUENCES TO PROTEINS INVOLVED IN TOCOPHEROL SYNTHESIS

INTRODUCTION

This application claims the benefit of the filing date of the provisional Application U.S. Serial Number 60/129,899, filed April 15, 1999, and the provisional Application, U.S. Serial Number 60/146,461, filed July 30, 1999.

TECHNICAL FIELD

The present invention is directed to nucleic acid and amino acid sequences and constructs, and methods related thereto.

BACKGROUND

Isoprenoids are ubiquitous compounds found in all living organisms. Plants synthesize a diverse array of greater than 22,000 isoprenoids (Connolly and Hill (1992) *Dictionary of Terpenoids*, Chapman and Hall, New York, NY). In plants, isoprenoids play essential roles in particular cell functions such as production of sterols, contributing to eukaryotic membrane architecture, acyclic polyprenoids found in the side chain of ubiquinone and plastoquinone, growth regulators like abscisic acid, gibberellins, brassinosteroids or the photosynthetic pigments chlorophylls and carotenoids. Although the physiological role of other plant isoprenoids is less evident, like that of the vast array of secondary metabolites, some are known to play key roles mediating the adaptative responses to different environmental challenges. In spite of the remarkable diversity of structure and function, all isoprenoids originate from a single metabolic precursor, isopentenyl diphosphate (IPP) (Wright, (1961) *Annu. Rev. Biochem.* 20:525-548; and Spurgeon and Porter, (1981) in Biosynthesis of Isoprenoid Compounds., Porter and Spurgeon eds (John Wiley, New York) Vol. 1, pp1-46).

A number of unique and interconnected biochemical pathways derived from the isoprenoid pathway leading to secondary metabolites, including tocopherols, exist in chloroplasts of higher plants. Tocopherols not only perform vital functions in plants, but are

also important from mammalian nutritional perspectives. In plastids, tocopherols account for up to 40% of the total quinone pool.

Tocopherols and tocotrienols (unsaturated tocopherol derivatives) are well known antioxidants, and play an important role in protecting cells from free radical damage, and in the prevention of many diseases, including cardiac disease, cancer, cataracts, retinopathy, Alzheimer's disease, and neurodegeneration, and have been shown to have beneficial effects on symptoms of arthritis, and in anti-aging. Vitamin E is used in chicken feed for improving the shelf life, appearance, flavor, and oxidative stability of meat, and to transfer tocols from feed to eggs. Vitamin E has been shown to be essential for normal reproduction, improves overall performance, and enhances immunocompetence in livestock animals. Vitamin E supplement in animal feed also imparts oxidative stability to milk products.

The demand for natural tocopherols as supplements has been steadily growing at a rate of 10-20% for the past three years. At present, the demand exceeds the supply for natural tocopherols, which are known to be more biopotent than racemic mixtures of synthetically produced tocopherols. Naturally occurring tocopherols are all *d*-stereomers, whereas synthetic α-tocopherol is a mixture of eight *d,l*-α-tocopherol isomers, only one of which (12.5%) is identical to the natural *d*-α-tocopherol. Natural *d*-α-tocopherol has the highest vitamin E activity (1.49 IU/mg) when compared to other natural tocopherols or tocotrienols. The synthetic α-tocopherol has a vitamin E activity of 1.1 IU/mg. In 1995, the worldwide market for raw refined tocopherols was $1020 million; synthetic materials comprised 85-88% of the market, the remaining 12-15% being natural materials. The best sources of natural tocopherols and tocotrienols are vegetable oils and grain products. Currently, most of the natural Vitamin E is produced from γ-tocopherol derived from soy oil processing, which is subsequently converted to α-tocopherol by chemical modification (α-tocopherol exhibits the greatest biological activity).

Methods of enhancing the levels of tocopherols and tocotrienols in plants, especially levels of the more desirable compounds that can be used directly, without chemical modification, would be useful to the art as such molecules exhibit better functionality and biovailability.

In addition, methods for the increased production of other isoprenoid derived compounds in a host plant cell is desirable. Furthermore, methods for the production of particular isoprenoid compounds in a host plant cell is also needed.

SUMMARY OF THE INVENTION

The present invention is directed to prenyltransferase (PT), and in particular to PT polynucleotides and polypeptides. The polynucleotides and polypeptides of the present invention include those derived from prokaryotic and eukaryotic sources.

Thus, one aspect of the present invention relates to isolated polynucleotide sequences encoding prenyltransferase proteins. In particular, isolated nucleic acid sequences encoding PT proteins from bacterial and plant sources are provided.

Another aspect of the present invention relates to oligonucleotides which include partial or complete PT encoding sequences.

It is also an aspect of the present invention to provide recombinant DNA constructs which can be used for transcription or transcription and translation (expression) of prenyltransferase. In particular, constructs are provided which are capable of transcription or transcription and translation in host cells.

In another aspect of the present invention, methods are provided for production of prenyltransferase in a host cell or progeny thereof. In particular, host cells are transformed or transfected with a DNA construct which can be used for transcription or transcription and translation of prenyltransferase. The recombinant cells which contain prenyltransferase are also part of the present invention.

In a further aspect, the present invention relates to methods of using polynucleotide and polypeptide sequences to modify the tocopherol content of host cells, particularly in host plant cells. Plant cells having such a modified tocopherol content are also contemplated herein.

The modified plants, seeds and oils obtained by the expression of the prenyltransferases are also considered part of the invention.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 provides an amino acid sequence alignment between ATPT2, ATPT3, ATPT4, ATPT8, and ATPT12 are performed using ClustalW.

Figure 2 provides a schematic picture of the expression construct pCGN10800.

Figure 3 provides a schematic picture of the expression construct pCGN10801.

Figure 4 provides a schematic picture of the expression construct pCGN10803.

Figure 5 provides a schematic picture of the expression construct pCGN10806.

Figure 6 provides a schematic picture of the expression construct pCGN10807.

Figure 7 provides a schematic picture of the expression construct pCGN10808.

Figure 8 provides a schematic picture of the expression construct pCGN10809.

Figure 9 provides a schematic picture of the expression construct pCGN10810.

Figure 10 provides a schematic picture of the expression construct pCGN10811.

Figure 11 provides a schematic picture of the expression construct pCGN10812.

Figure 12 provides a schematic picture of the expression construct pCGN10813.

Figure 13 provides a schematic picture of the expression construct pCGN10814.

Figure 14 provides a schematic picture of the expression construct pCGN10815.

Figure 15 provides a schematic picture of the expression construct pCGN10816.

Figure 16 provides a schematic picture of the expression construct pCGN10817.

Figure 17 provides a schematic picture of the expression construct pCGN10819.

Figure 18 provides a schematic picture of the expression construct pCGN10824.

Figure 19 provides a schematic picture of the expression construct pCGN10825.

Figure 20 provides a schematic picture of the expression construct pCGN10826.

Figure 21 provides an amino acid sequence alignment using ClustalW between the *Synechocystis* sequence knockouts.

Figure 22 provides an amino acid sequence of the ATPT2, ATPT3, ATPT4, ATPT8, and ATPT12 protein sequences from *Arabidopsis* and the slr1736, slr0926, sll1899, slr0056, and the slr1518 amino acid sequences from *Synechocystis*.

Figure 23 provides the results of the enzymatic assay from preparations of wild type *Synechocystis* strain 6803, and *Synechocystis* slr1736 knockout.

Figure 24 provides bar graphs of HPLC data obtained from seed extracts of transgenic *Arabidopsis* containing pCGN10822, which provides of the expression of the ATPT2 sequence, in the sense orientation, from the napin promoter. Provided are graphs for alpha, gamma, and delta tocopherols, as well as total tocopherol for 22 transformed lines, as well as a nontransformed (wildtype) control.

Figure 25 provides a bar graph of HPLC analysis of seed extracts from *Arabidopsis* plants transformed with pCGN10803 (35S-ATPT2, in the antisense orientation), pCGN10802

(line 1625, napin ATPT2 in the sense orientation), pCGN10809 (line 1627, 35S-ATPT3 in the sense orientation), a nontransformed (wt) constrol, and a empty vector transformed control.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides, *inter alia*, compositions and methods for altering (for example, increasing and decreasing) the tocopherol levels and/or modulating their ratios in host cells. In particular, the present invention provides polynucleotides, polypeptides, and methods of use thereof for the modulation of tocopherol content in host plant cells.

The present invention provides polynucleotide and polypeptide sequences involved in the prenylation of straight chain and aromatic compounds. Straight chain prenyl transferases as used herein comprises sequences which encode proteins involved in the prenylation of straight chain compounds, including, but not limited to, geranyl geranyl pyrophosphate and farnesyl pyrophosphate. Aromatic prenyl transferases, as used herein, comprises sequences which encode proteins involved in the prenylation of aromatic compounds, including, but not limited to, menaquinone, ubiquinone, chlorophyll, and homogentisic acid. The prenyl transferase of the present invention preferably prenylates homogentisic acid.

The biosynthesis of α-tocopherol in higher plants involves condensation of homogentisic acid and phytylpyrophosphate to form 2-methyl-6 phytylbenzoquinol that can, by cyclization and subsequent methylations (Fiedler et al., 1982, *Planta*, 155: 511-515, Soll et al., 1980, *Arch. Biochem. Biophys*. 204: 544-550, Marshall et al., 1985 *Phytochem.*, 24: 1705-1711, all of which are herein incorporated by reference in their entirety), form various tocopherols. The *Arabidopsis pds2* mutant identified and characterized by Norris *et al.* (1995), is deficient in tocopherol and plastiquinone-9 accumulation. Further genetic and biochemical analysis suggests that the protein encoded by *PDS2* may be responsible for the prenylation of homogentisic acid. This may be a rate limiting step in tocopherol biosynthesis, and this gene has yet to be isolated. Thus, it is an aspect of the present invention to provide polynucleotides and polypeptides involved in the prenylation of homogentisic acid.

Isolated Polynucleotides, Proteins, and Polypeptides

A first aspect of the present invention relates to isolated prenyltransferase polynucleotides. The polynucleotide sequences of the present invention include isolated polynucleotides that encode the polypeptides of the invention having a deduced amino acid sequence selected from the group of sequences set forth in the Sequence Listing and to other polynucleotide sequences closely related to such sequences and variants thereof.

The invention provides a polynucleotide sequence identical over its entire length to each coding sequence as set forth in the Sequence Listing. The invention also provides the coding sequence for the mature polypeptide or a fragment thereof, as well as the coding sequence for the mature polypeptide or a fragment thereof in a reading frame with other coding sequences, such as those encoding a leader or secretory sequence, a pre-, pro-, or prepro- protein sequence. The polynucleotide can also include non-coding sequences, including for example, but not limited to, non-coding 5' and 3' sequences, such as the transcribed, untranslated sequences, termination signals, ribosome binding sites, sequences that stabilize mRNA, introns, polyadenylation signals, and additional coding sequence that encodes additional amino acids. For example, a marker sequence can be included to facilitate the purification of the fused polypeptide. Polynucleotides of the present invention also include polynucleotides comprising a structural gene and the naturally associated sequences that control gene expression.

The invention also includes polynucleotides of the formula:

$$X\text{-}(R_1)_n\text{-}(R_2)\text{-}(R_3)_n\text{-}Y$$

wherein, at the 5' end, X is hydrogen, and at the 3' end, Y is hydrogen or a metal, R_1 and R_3 are any nucleic acid residue, n is an integer between 1 and 3000, preferably between 1 and 1000 and R_2 is a nucleic acid sequence of the invention, particularly a nucleic acid sequence selected from the group set forth in the Sequence Listing and preferably those of SEQ ID NOs: 1, 3, 5, 7, 8, 10, 11, 13-16, 18, 23, 29, 36, and 38. In the formula, R_2 is oriented so that its 5' end residue is at the left, bound to R_1, and its 3' end residue is at the right, bound to R_3. Any stretch of nucleic acid residues denoted by either R group, where R is greater than 1, may be either a heteropolymer or a homopolymer, preferably a heteropolymer.

The invention also relates to variants of the polynucleotides described herein that encode for variants of the polypeptides of the invention. Variants that are fragments of the polynucleotides of the invention can be used to synthesize full-length polynucleotides of the invention. Preferred embodiments are polynucleotides encoding polypeptide variants wherein

5 to 10, 1 to 5, 1 to 3, 2, 1 or no amino acid residues of a polypeptide sequence of the invention are substituted, added or deleted, in any combination. Particularly preferred are substitutions, additions, and deletions that are silent such that they do not alter the properties or activities of the polynucleotide or polypeptide.

Further preferred embodiments of the invention that are at least 50%, 60%, or 70% identical over their entire length to a polynucleotide encoding a polypeptide of the invention, and polynucleotides that are complementary to such polynucleotides. More preferable are polynucleotides that comprise a region that is at least 80% identical over its entire length to a polynucleotide encoding a polypeptide of the invention and polynucleotides that are complementary thereto. In this regard, polynucleotides at least 90% identical over their entire length are particularly preferred, those at least 95% identical are especially preferred. Further, those with at least 97% identity are highly preferred and those with at least 98% and 99% identity are particularly highly preferred, with those at least 99% being the most highly preferred.

Preferred embodiments are polynucleotides that encode polypeptides that retain substantially the same biological function or activity as the mature polypeptides encoded by the polynucleotides set forth in the Sequence Listing.

The invention further relates to polynucleotides that hybridize to the above-described sequences. In particular, the invention relates to polynucleotides that hybridize under stringent conditions to the above-described polynucleotides. As used herein, the terms "stringent conditions" and "stringent hybridization conditions" mean that hybridization will generally occur if there is at least 95% and preferably at least 97% identity between the sequences. An example of stringent hybridization conditions is overnight incubation at 42°C in a solution comprising 50% formamide, 5x SSC (150 mM NaCl, 15 mM trisodium citrate), 50 mM sodium phosphate (pH 7.6), 5x Denhardt's solution, 10% dextran sulfate, and 20 micrograms/milliliter denatured, sheared salmon sperm DNA, followed by washing the hybridization support in 0.1x SSC at approximately 65°C. Other hybridization and wash conditions are well known and are exemplified in Sambrook, *et al.*, Molecular Cloning: A Laboratory Manual, Second Edition, cold Spring Harbor, NY (1989), particularly Chapter 11.

The invention also provides a polynucleotide consisting essentially of a polynucleotide sequence obtainable by screening an appropriate library containing the complete gene for a polynucleotide sequence set for in the Sequence Listing under stringent hybridization conditions with a probe having the sequence of said polynucleotide sequence or

a fragment thereof; and isolating said polynucleotide sequence. Fragments useful for obtaining such a polynucleotide include, for example, probes and primers as described herein.

As discussed herein regarding polynucleotide assays of the invention, for example, polynucleotides of the invention can be used as a hybridization probe for RNA, cDNA, or genomic DNA to isolate full length cDNAs or genomic clones encoding a polypeptide and to isolate cDNA or genomic clones of other genes that have a high sequence similarity to a polynucleotide set forth in the Sequence Listing. Such probes will generally comprise at least 15 bases. Preferably such probes will have at least 30 bases and can have at least 50 bases. Particularly preferred probes will have between 30 bases and 50 bases, inclusive.

The coding region of each gene that comprises or is comprised by a polynucleotide sequence set forth in the Sequence Listing may be isolated by screening using a DNA sequence provided in the Sequence Listing to synthesize an oligonucleotide probe. A labeled oligonucleotide having a sequence complementary to that of a gene of the invention is then used to screen a library of cDNA, genomic DNA or mRNA to identify members of the library which hybridize to the probe. For example, synthetic oligonucleotides are prepared which correspond to the prenyltransferase EST sequences. The oligonucleotides are used as primers in polymerase chain reaction (PCR) techniques to obtain 5' and 3' terminal sequence of prenyl transferase genes. Alternatively, where oligonucleotides of low degeneracy can be prepared from particular prenyltransferase peptides, such probes may be used directly to screen gene libraries for prenyltransferase gene sequences. In particular, screening of cDNA libraries in phage vectors is useful in such methods due to lower levels of background hybridization.

Typically, a prenyltransferase sequence obtainable from the use of nucleic acid probes will show 60-70% sequence identity between the target prenyltransferase sequence and the encoding sequence used as a probe. However, lengthy sequences with as little as 50-60% sequence identity may also be obtained. The nucleic acid probes may be a lengthy fragment of the nucleic acid sequence, or may also be a shorter, oligonucleotide probe. When longer nucleic acid fragments are employed as probes (greater than about 100 bp), one may screen at lower stringencies in order to obtain sequences from the target sample which have 20-50% deviation (i.e., 50-80% sequence homology) from the sequences used as probe.

Oligonucleotide probes can be considerably shorter than the entire nucleic acid sequence encoding an prenyltransferase enzyme, but should be at least about 10, preferably at least about 15, and more preferably at least about 20 nucleotides. A higher degree of sequence

identity is desired when shorter regions are used as opposed to longer regions. It may thus be desirable to identify regions of highly conserved amino acid sequence to design oligonucleotide probes for detecting and recovering other related prenyltransferase genes. Shorter probes are often particularly useful for polymerase chain reactions (PCR), especially when highly conserved sequences can be identified. (*See*, Gould, *et al.*, *PNAS USA* (1989) *86*:1934-1938.).

Another aspect of the present invention relates to prenyltransferase polypeptides. Such polypeptides include isolated polypeptides set forth in the Sequence Listing, as well as polypeptides and fragments thereof, particularly those polypeptides which exhibit prenyltransferase activity and also those polypeptides which have at least 50%, 60% or 70% identity, preferably at least 80% identity, more preferably at least 90% identity, and most preferably at least 95% identity to a polypeptide sequence selected from the group of sequences set forth in the Sequence Listing, and also include portions of such polypeptides, wherein such portion of the polypeptide preferably includes at least 30 amino acids and more preferably includes at least 50 amino acids.

"Identity", as is well understood in the art, is a relationship between two or more polypeptide sequences or two or more polynucleotide sequences, as determined by comparing the sequences. In the art, "identity" also means the degree of sequence relatedness between polypeptide or polynucleotide sequences, as determined by the match between strings of such sequences. "Identity" can be readily calculated by known methods including, but not limited to, those described in *Computational Molecular Biology*, Lesk, A.M., ed., Oxford University Press, New York (1988); *Biocomputing: Informatics and Genome Projects*, Smith, D.W., ed., Academic Press, New York, 1993; *Computer Analysis of Sequence Data, Part I*, Griffin, A.M. and Griffin, H.G., eds., Humana Press, New Jersey (1994); *Sequence Analysis in Molecular Biology*, von Heinje, G., Academic Press (1987); *Sequence Analysis Primer*, Gribskov, M. and Devereux, J., eds., Stockton Press, New York (1991); and Carillo, H., and Lipman, D., SIAM *J Applied Math*, 48:1073 (1988). Methods to determine identity are designed to give the largest match between the sequences tested. Moreover, methods to determine identity are codified in publicly available programs. Computer programs which can be used to determine identity between two sequences include, but are not limited to, GCG (Devereux, J., et al., *Nucleic Acids Research* 12(1):387 (1984); suite of five BLAST programs, three designed for nucleotide sequences queries (BLASTN, BLASTX, and TBLASTX) and two designed for protein sequence queries (BLASTP and TBLASTN)

(Coulson, *Trends in Biotechnology, 12:* 76-80 (1994); Birren, *et al., Genome Analysis, 1:* 543-559 (1997)). The BLAST X program is publicly available from NCBI and other sources (*BLAST Manual*, Altschul, S., *et al.*, NCBI NLM NIH, Bethesda, MD 20894; Altschul, S., *et al., J. Mol. Biol.*, 215:403-410 (1990)). The well known Smith Waterman algorithm can also be used to determine identity.

Parameters for polypeptide sequence comparison typically include the following:

Algorithm: Needleman and Wunsch, *J. Mol. Biol.* 48:443-453 (1970)

Comparison matrix: BLOSSUM62 from Hentikoff and Hentikoff, *Proc. Natl. Acad. Sci USA* 89:10915-10919 (1992)

Gap Penalty: 12

Gap Length Penalty: 4

A program which can be used with these parameters is publicly available as the "gap" program from Genetics Computer Group, Madison Wisconsin. The above parameters along with no penalty for end gap are the default parameters for peptide comparisons.

Parameters for polynucleotide sequence comparison include the following:

Algorithm: Needleman and Wunsch, J. Mol. Biol. 48:443-453 (1970)

Comparison matrix: matches = +10; mismatches = 0

Gap Penalty: 50

Gap Length Penalty: 3

A program which can be used with these parameters is publicly available as the "gap" program from Genetics Computer Group, Madison Wisconsin. The above parameters are the default parameters for nucleic acid comparisons.

The invention also includes polypeptides of the formula:

$$X\text{-}(R_1)_n\text{-}(R_2)\text{-}(R_3)_n\text{-}Y$$

wherein, at the amino terminus, X is hydrogen, and at the carboxyl terminus, Y is hydrogen or a metal, R_1 and R_3 are any amino acid residue, n is an integer between 1 and 1000, and R_2 is an amino acid sequence of the invention, particularly an amino acid sequence selected from the group set forth in the Sequence Listing and preferably those encoded by the sequences provided in SEQ ID NOs: 2, 4, 6, 9, 12, 17, 19-22, 24-28, 30, 32-35, 37, and 39. In the formula, R_2 is oriented so that its amino terminal residue is at the left, bound to R_1, and its carboxy terminal residue is at the right, bound to R_3. Any stretch of amino acid residues denoted by either R group, where R is greater than 1, may be either a heteropolymer or a homopolymer, preferably a heteropolymer.

Polypeptides of the present invention include isolated polypeptides encoded by a polynucleotide comprising a sequence selected from the group of a sequence contained in the Sequence Listing set forth herein .

The polypeptides of the present invention can be mature protein or can be part of a fusion protein.

Fragments and variants of the polypeptides are also considered to be a part of the invention. A fragment is a variant polypeptide which has an amino acid sequence that is entirely the same as part but not all of the amino acid sequence of the previously described polypeptides. The fragments can be "free-standing" or comprised within a larger polypeptide of which the fragment forms a part or a region, most preferably as a single continuous region. Preferred fragments are biologically active fragments which are those fragments that mediate activities of the polypeptides of the invention, including those with similar activity or improved activity or with a decreased activity. Also included are those fragments that antigenic or immunogenic in an animal, particularly a human.

Variants of the polypeptide also include polypeptides that vary from the sequences set forth in the Sequence Listing by conservative amino acid substitutions, substitution of a residue by another with like characteristics. In general, such substitutions are among Ala, Val, Leu and Ile; between Ser and Thr; between Asp and Glu; between Asn and Gln; between Lys and Arg; or between Phe and Tyr. Particularly preferred are variants in which 5 to 10; 1 to 5; 1 to 3 or one amino acid(s) are substituted, deleted, or added, in any combination.

Variants that are fragments of the polypeptides of the invention can be used to produce the corresponding full length polypeptide by peptide synthesis. Therefore, these variants can be used as intermediates for producing the full-length polypeptides of the invention.

The polynucleotides and polypeptides of the invention can be used, for example, in the transformation of host cells, such as plant host cells, as further discussed herein.

The invention also provides polynucleotides that encode a polypeptide that is a mature protein plus additional amino or carboxyl-terminal amino acids, or amino acids within the mature polypeptide (for example, when the mature form of the protein has more than one polypeptide chain). Such sequences can, for example, play a role in the processing of a protein from a precursor to a mature form, allow protein transport, shorten or lengthen protein half-life, or facilitate manipulation of the protein in assays or production. It is contemplated

that cellular enzymes can be used to remove any additional amino acids from the mature protein.

A precursor protein, having the mature form of the polypeptide fused to one or more prosequences may be an inactive form of the polypeptide. The inactive precursors generally are activated when the prosequences are removed. Some or all of the prosequences may be removed prior to activation. Such precursor protein are generally called proproteins.

Plant Constructs and Methods of Use

Of particular interest is the use of the nucleotide sequences in recombinant DNA constructs to direct the transcription or transcription and translation (expression) of the prenyltransferase sequences of the present invention in a host plant cell. The expression constructs generally comprise a promoter functional in a host plant cell operably linked to a nucleic acid sequence encoding a prenyltransferase of the present invention and a transcriptional termination region functional in a host plant cell.

A first nucleic acid sequence is "operably linked" or "operably associated" with a second nucleic acid sequence when the sequences are so arranged that the first nucleic acid sequence affects the function of the second nucleic-acid sequence. Preferably, the two sequences are part of a single contiguous nucleic acid molecule and more preferably are adjacent. For example, a promoter is operably linked to a gene if the promoter regulates or mediates transcription of the gene in a cell.

Those skilled in the art will recognize that there are a number of promoters which are functional in plant cells, and have been described in the literature. Chloroplast and plastid specific promoters, chloroplast or plastid functional promoters, and chloroplast or plastid operable promoters are also envisioned.

One set of plant functional promoters are constitutive promoters such as the CaMV35S or FMV35S promoters that yield high levels of expression in most plant organs. Enhanced or duplicated versions of the CaMV35S and FMV35S promoters are useful in the practice of this invention (Odell, *et al.* (1985) *Nature* 313:810-812; Rogers, U.S. Patent Number 5,378, 619). In addition, it may also be preferred to bring about expression of the prenyltransferase gene in specific tissues of the plant, such as leaf, stem, root, tuber, seed, fruit, etc., and the promoter chosen should have the desired tissue and developmental specificity.

Of particular interest is the expression of the nucleic acid sequences of the present invention from transcription initiation regions which are preferentially expressed in a plant seed tissue. Examples of such seed preferential transcription initiation sequences include those sequences derived from sequences encoding plant storage protein genes or from genes involved in fatty acid biosynthesis in oilseeds. Examples of such promoters include the 5' regulatory regions from such genes as napin (Kridl *et al.*, *Seed Sci. Res. 1*:209:219 (1991)), phaseolin, zein, soybean trypsin inhibitor, ACP, stearoyl-ACP desaturase, soybean α' subunit of β-conglycinin (soy 7s, (Chen *et al.*, *Proc. Natl. Acad. Sci.*, 83:8560-8564 (1986))) and oleosin.

It may be advantageous to direct the localization of proteins conferring prenyltransferase to a particular subcellular compartment, for example, to the mitochondrion, endoplasmic reticulum, vacuoles, chloroplast or other plastidic compartment. For example, where the genes of interest of the present invention will be targeted to plastids, such as chloroplasts, for expression, the constructs will also employ the use of sequences to direct the gene to the plastid. Such sequences are referred to herein as chloroplast transit peptides (CTP) or plastid transit peptides (PTP). In this manner, where the gene of interest is not directly inserted into the plastid, the expression construct will additionally contain a gene encoding a transit peptide to direct the gene of interest to the plastid. The chloroplast transit peptides may be derived from the gene of interest, or may be derived from a heterologous sequence having a CTP. Such transit peptides are known in the art. See, for example, Von Heijne *et al.* (1991) *Plant Mol. Biol. Rep. 9*:104-126; Clark *et al.* (1989) *J. Biol. Chem. 264*:17544-17550; della-Cioppa *et al.* (1987) *Plant Physiol. 84*:965-968; Romer *et al.* (1993) *Biochem. Biophys. Res Commun. 196*:1414-1421; and, Shah *et al.* (1986) *Science 233*:478-481.

Depending upon the intended use, the constructs may contain the nucleic acid sequence which encodes the entire prenyltransferase protein, or a portion thereof. For example, where antisense inhibition of a given prenyltransferase protein is desired, the entire prenyltransferase sequence is not required. Furthermore, where prenyltransferase sequences used in constructs are intended for use as probes, it may be advantageous to prepare constructs containing only a particular portion of a prenyltransferase encoding sequence, for example a sequence which is discovered to encode a highly conserved prenyltransferase region.

The skilled artisan will recognize that there are various methods for the inhibition of expression of endogenous sequences in a host cell. Such methods include, but are not limited to, antisense suppression (Smith, *et al.* (1988) *Nature* 334:724-726) , co-suppression (Napoli, *et al.* (1989) *Plant Cell* 2:279-289), ribozymes (PCT Publication WO 97/10328), and combinations of sense and antisense Waterhouse, *et al.* (1998) *Proc. Natl. Acad. Sci. USA* 95:13959-13964. Methods for the suppression of endogenous sequences in a host cell typically employ the transcription or transcription and translation of at least a portion of the sequence to be suppressed. Such sequences may be homologous to coding as well as non-coding regions of the endogenous sequence.

Regulatory transcript termination regions may be provided in plant expression constructs of this invention as well. Transcript termination regions may be provided by the DNA sequence encoding the prenyltransferase or a convenient transcription termination region derived from a different gene source, for example, the transcript termination region which is naturally associated with the transcript initiation region. The skilled artisan will recognize that any convenient transcript termination region which is capable of terminating transcription in a plant cell may be employed in the constructs of the present invention.

Alternatively, constructs may be prepared to direct the expression of the prenyltransferase sequences directly from the host plant cell plastid. Such constructs and methods are known in the art and are generally described, for example, in Svab, *et al.* (1990) *Proc. Natl. Acad. Sci. USA* 87:8526-8530 and Svab and Maliga (1993) *Proc. Natl. Acad. Sci. USA* 90:913-917 and in U.S. Patent Number 5,693,507.

The prenyltransferase constructs of the present invention can be used in transformation methods with additional constructs providing for the expression of other nucleic acid sequences encoding proteins involved in the production of tocopherols, or tocopherol precursors such as homogentisic acid and/or phytylpyrophosphate. Nucleic acid sequences encoding proteins involved in the production of homogentisic acid are known in the art, and include but not are limited to, 4-hydroxyphenylpyruvate dioxygenase (HPPD, EC 1.13.11.27) described for example, by Garcia, *et al.* ((1999) *Plant Physiol.* 119(4):1507-1516), mono or bifunctional tyrA (described for example by Xia, *et al.* (1992) *J. Gen Microbiol.* 138:1309-1316, and Hudson, *et al.* (1984) *J. Mol. Biol.* 180:1023-1051), Oxygenase, 4-hydroxyphenylpyruvate di- (9CI), 4-Hydroxyphenylpyruvate dioxygenase; p-Hydroxyphenylpyruvate dioxygenase; p-Hydroxyphenylpyruvate hydroxylase; p-Hydroxyphenylpyruvate oxidase; p-Hydroxyphenylpyruvic acid hydroxylase; p-

Hydroxyphenylpyruvic hydroxylase; p-Hydroxyphenylpyruvic oxidase), 4-hydroxyphenylacetate, NAD(P)H:oxygen oxidoreductase (1-hydroxylating); 4-hydroxyphenylacetate 1-monooxygenase, and the like. In addition, constructs for the expression of nucleic acid sequences encoding proteins involved in the production of phytylpyrophosphate can also be employed with the prenyltransferase constructs of the present invention. Nucleic acid sequences encoding proteins involved in the production of phytylpyrophosphate are known in the art, and include, but are not limited to geranylgeranylpyrophosphate synthase (GGPPS), geranylgeranylpyrophosphate reductase (GGH), 1-deoxyxylulose-5-phosphate synthase, 1- deoxy-D-xylolose-5-phosphate reductoisomerase, 4-diphosphocytidyl-2-C-methylerythritol synthase, isopentyl pyrophosphate isomerase.

The prenyltransferase sequences of the present invention find use in the preparation of transformation constructs having a second expression cassette for the expression of additional sequences involved in tocopherol biosynthesis. Additional tocopherol biosynthesis sequences of interest in the present invention include, but are not limited to gamma-tocpherol methyltransferase (Shintani, *et al.* (1998) *Science* 282(5396):2098-2100), tocopherol cyclase, and tocopherol methyltransferase.

A plant cell, tissue, organ, or plant into which the recombinant DNA constructs containing the expression constructs have been introduced is considered transformed, transfected, or transgenic. A transgenic or transformed cell or plant also includes progeny of the cell or plant and progeny produced from a breeding program employing such a transgenic plant as a parent in a cross and exhibiting an altered phenotype resulting from the presence of a prenyltransferase nucleic acid sequence.

Plant expression or transcription constructs having a prenyltransferase as the DNA sequence of interest for increased or decreased expression thereof may be employed with a wide variety of plant life, particularly, plant life involved in the production of vegetable oils for edible and industrial uses. Particularly preferred plants for use in the methods of the present invention include, but are not limited to: *Acacia,* alfalfa, aneth, apple, apricot, artichoke, arugula, asparagus, avocado, banana, barley, beans, beet, blackberry, blueberry, broccoli, brussels sprouts, cabbage, canola, cantaloupe, carrot, cassava, cauliflower, celery, cherry, chicory, cilantro, citrus, clementines, coffee, corn, cotton, cucumber, Douglas fir, eggplant, endive, escarole, eucalyptus, fennel, figs, garlic, gourd, grape, grapefruit, honey dew, jicama, kiwifruit, lettuce, leeks, lemon, lime, Loblolly pine, mango, melon, mushroom,

nectarine, nut, oat, oil palm, oil seed rape, okra, onion, orange, an ornamental plant, papaya, parsley, pea, peach, peanut, pear, pepper, persimmon, pine, pineapple, plantain, plum, pomegranate, poplar, potato, pumpkin, quince, radiata pine, radicchio, radish, raspberry, rice, rye, sorghum, Southern pine, soybean, spinach, squash, strawberry, sugarbeet, sugarcane, sunflower, sweet potato, sweetgum, tangerine, tea, tobacco, tomato, triticale, turf, turnip, a vine, watermelon, wheat, yams, and zucchini.

Most especially preferred are temperate oilseed crops. Temperate oilseed crops of interest include, but are not limited to, rapeseed (Canola and High Erucic Acid varieties), sunflower, safflower, cotton, soybean, peanut, coconut and oil palms, and corn. Depending on the method for introducing the recombinant constructs into the host cell, other DNA sequences may be required. Importantly, this invention is applicable to dicotyledyons and monocotyledons species alike and will be readily applicable to new and/or improved transformation and regulation techniques.

Of particular interest, is the use of prenyltransferase constructs in plants to produce plants or plant parts, including, but not limited to leaves, stems, roots, reproductive, and seed, with a modified content of tocopherols in plant parts having transformed plant cells.

For immunological screening, antibodies to the protein can be prepared by injecting rabbits or mice with the purified protein or portion thereof, such methods of preparing antibodies being well known to those in the art. Either monoclonal or polyclonal antibodies can be produced, although typically polyclonal antibodies are more useful for gene isolation. Western analysis may be conducted to determine that a related protein is present in a crude extract of the desired plant species, as determined by cross-reaction with the antibodies to the encoded proteins. When cross-reactivity is observed, genes encoding the related proteins are isolated by screening expression libraries representing the desired plant species. Expression libraries can be constructed in a variety of commercially available vectors, including lambda gt11, as described in Sambrook, *et al.* (*Molecular Cloning: A Laboratory Manual*, Second Edition (1989) Cold Spring Harbor Laboratory, Cold Spring Harbor, New York).

To confirm the activity and specificity of the proteins encoded by the identified nucleic acid sequences as prenyltransferase enzymes, *in vitro* assays are performed in insect cell cultures using baculovirus expression systems. Such baculovirus expression systems are known in the art and are described by Lee, *et al.* U.S. Patent Number 5,348,886, the entirety of which is herein incorporated by reference.

In addition, other expression constructs may be prepared to assay for protein activity utilizing different expression systems. Such expression constructs are transformed into yeast or prokaryotic host and assayed for prenyltransferase activity. Such expression systems are known in the art and are readily available through commercial sources.

In addition to the sequences described in the present invention, DNA coding sequences useful in the present invention can be derived from algae, fungi, bacteria, mammalian sources, plants, etc. Homology searches in existing databases using signature sequences corresponding to conserved nucleotide and amino acid sequences of prenyltransferase can be employed to isolate equivalent, related genes from other sources such as plants and microorganisms. Searches in EST databases can also be employed. Furthermore, the use of DNA sequences encoding enzymes functionally enzymatically equivalent to those disclosed herein, wherein such DNA sequences are degenerate equivalents of the nucleic acid sequences disclosed herein in accordance with the degeneracy of the genetic code, is also encompassed by the present invention. Demonstration of the functionality of coding sequences identified by any of these methods can be carried out by complementation of mutants of appropriate organisms, such as *Synechocystis, Shewanella, yeast, Pseudomonas, Rhodobacteria*, etc., that lack specific biochemical reactions, or that have been mutated. The sequences of the DNA coding regions can be optimized by gene resynthesis, based on codon usage, for maximum expression in particular hosts.

For the alteration of tocopherol production in a host cell, a second expression construct can be used in accordance with the present invention. For example, the prenyltransferase expression construct can be introduced into a host cell in conjunction with a second expression construct having a nucleotide sequence for a protein involved in tocopherol biosynthesis.

The method of transformation in obtaining such transgenic plants is not critical to the instant invention, and various methods of plant transformation are currently available. Furthermore, as newer methods become available to transform crops, they may also be directly applied hereunder. For example, many plant species naturally susceptible to *Agrobacterium* infection may be successfully transformed via tripartite or binary vector methods of *Agrobacterium* mediated transformation. In many instances, it will be desirable to have the construct bordered on one or both sides by T-DNA, particularly having the left and right borders, more particularly the right border. This is particularly useful when the construct uses *A. tumefaciens* or *A. rhizogenes* as a mode for transformation, although the T-

DNA borders may find use with other modes of transformation. In addition, techniques of microinjection, DNA particle bombardment, and electroporation have been developed which allow for the transformation of various monocot and dicot plant species.

Normally, included with the DNA construct will be a structural gene having the necessary regulatory regions for expression in a host and providing for selection of transformant cells. The gene may provide for resistance to a cytotoxic agent, e.g. antibiotic, heavy metal, toxin, etc., complementation providing prototrophy to an auxotrophic host, viral immunity or the like. Depending upon the number of different host species the expression construct or components thereof are introduced, one or more markers may be employed, where different conditions for selection are used for the different hosts.

Where *Agrobacterium* is used for plant cell transformation, a vector may be used which may be introduced into the *Agrobacterium* host for homologous recombination with T-DNA or the Ti- or Ri-plasmid present in the *Agrobacterium* host. The Ti- or Ri-plasmid containing the T-DNA for recombination may be armed (capable of causing gall formation) or disarmed (incapable of causing gall formation), the latter being permissible, so long as the *vir* genes are present in the transformed *Agrobacterium* host. The armed plasmid can give a mixture of normal plant cells and gall.

In some instances where *Agrobacterium* is used as the vehicle for transforming host plant cells, the expression or transcription construct bordered by the T-DNA border region(s) will be inserted into a broad host range vector capable of replication in *E. coli* and *Agrobacterium*, there being broad host range vectors described in the literature. Commonly used is pRK2 or derivatives thereof. See, for example, Ditta, *et al.*, (*Proc. Nat. Acad. Sci., U.S.A.* (1980) *77*:7347-7351) and EPA 0 120 515, which are incorporated herein by reference. Alternatively, one may insert the sequences to be expressed in plant cells into a vector containing separate replication sequences, one of which stabilizes the vector in *E. coli*, and the other in *Agrobacterium*. See, for example, McBride, *et al.* (*Plant Mol. Biol.* (1990) *14*:269-276), wherein the pRiHRI (Jouanin, *et al.*, *Mol. Gen. Genet.* (1985) 201:370-374) origin of replication is utilized and provides for added stability of the plant expression vectors in host *Agrobacterium* cells.

Included with the expression construct and the T-DNA will be one or more markers, which allow for selection of transformed Agrobacterium and transformed plant cells. A number of markers have been developed for use with plant cells, such as resistance to chloramphenicol, kanamycin, the aminoglycoside G418, hygromycin, or the like. The

particular marker employed is not essential to this invention, one or another marker being preferred depending on the particular host and the manner of construction.

For transformation of plant cells using *Agrobacterium*, explants may be combined and incubated with the transformed *Agrobacterium* for sufficient time for transformation, the bacteria killed, and the plant cells cultured in an appropriate selective medium. Once callus forms, shoot formation can be encouraged by employing the appropriate plant hormones in accordance with known methods and the shoots transferred to rooting medium for regeneration of plants. The plants may then be grown to seed and the seed used to establish repetitive generations and for isolation of vegetable oils.

There are several possible ways to obtain the plant cells of this invention which contain multiple expression constructs. Any means for producing a plant comprising a construct having a DNA sequence encoding the expression construct of the present invention, and at least one other construct having another DNA sequence encoding an enzyme are encompassed by the present invention. For example, the expression construct can be used to transform a plant at the same time as the second construct either by inclusion of both expression constructs in a single transformation vector or by using separate vectors, each of which express desired genes. The second construct can be introduced into a plant which has already been transformed with the prenyltransferase expression construct, or alternatively, transformed plants, one expressing the prenyltransferase construct and one expressing the second construct, can be crossed to bring the constructs together in the same plant.

The nucleic acid sequences of the present invention can be used in constructs to provide for the expression of the sequence in a variety of host cells, both prokaryotic eukaryotic. Host cells of the present invention preferably include monocotyledenous and dicotyledenous plant cells.

In general, the skilled artisan is familiar with the standard resource materials which describe specific conditions and procedures for the construction, manipulation and isolation of macromolecules (e.g., DNA molecules, plasmids, etc.), generation of recombinant organisms and the screening and isolating of clones, (see for example, Sambrook *et al.*, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Press (1989); Maliga *et al.*, *Methods in Plant Molecular Biology,* Cold Spring Harbor Press (1995), the entirety of which is herein incorporated by reference; Birren *et al.*, *Genome Analysis: Analyzing DNA*, 1, Cold Spring Harbor, New York, the entirety of which is herein incorporated by reference).

Methods for the expression of sequences in insect host cells are known in the art. Baculovirus expression vectors are recombinant insect viruses in which the coding sequence for a chosen foreign gene has been inserted behind a baculovirus promoter in place of the viral gene, e.g., polyhedrin (Smith and Summers, U.S. Pat. No., 4,745,051, the entirety of which is incorporated herein by reference). Baculovirus expression vectors are known in the art, and are described for example in Doerfler, *Curr. Top. Microbiol. Immunol. 131*:51-68 (1968); Luckow and Summers, *Bio/Technology 6*:47-55 (1988a); Miller, *Annual Review of Microbiol. 42*:177-199 (1988); Summers, *Curr. Comm. Molecular Biology*, Cold Spring Harbor Press, Cold Spring Harbor, N.Y. (1988); Summers and Smith, *A Manual of Methods for Baculovirus Vectors and Insect Cell Culture Procedures,* Texas Ag. Exper. Station Bulletin No. 1555 (1988), the entireties of which is herein incorporated by reference)

Methods for the expression of a nucleic acid sequence of interest in a fungal host cell are known in the art. The fungal host cell may, for example, be a yeast cell or a filamentous fungal cell. Methods for the expression of DNA sequences of interest in yeast cells are generally described in "Guide to yeast genetics and molecular biology", Guthrie and Fink, eds. Methods in enzymology , Academic Press, Inc. Vol 194 (1991) and Gene expression technology", Goeddel ed, Methods in Enzymology, Academic Press, Inc., Vol 185 (1991).

Mammalian cell lines available as hosts for expression are known in the art and include many immortalized cell lines available from the American Type Culture Collection (ATCC, Manassas, VA), such as HeLa cells, Chinese hamster ovary (CHO) cells, baby hamster kidney (BHK) cells and a number of other cell lines. Suitable promoters for mammalian cells are also known in the art and include, but are not limited to, viral promoters such as that from Simian Virus 40 (SV40) (Fiers *et al., Nature* 273:113 (1978), the entirety of which is herein incorporated by reference), Rous sarcoma virus (RSV), adenovirus (ADV) and bovine papilloma virus (BPV). Mammalian cells may also require terminator sequences and poly-A addition sequences. Enhancer sequences which increase expression may also be included and sequences which promote amplification of the gene may also be desirable (for example methotrexate resistance genes).

Vectors suitable for replication in mammalian cells are well known in the art, and may include viral replicons, or sequences which insure integration of the appropriate sequences encoding epitopes into the host genome. Plasmid vectors that greatly facilitate the construction of recombinant viruses have been described (*see*, for example, Mackett *et al*, *J Virol. 49*:857 (1984); Chakrabarti *et al., Mol. Cell. Biol. 5*:3403 (1985); Moss, In: *Gene*

Transfer Vectors For Mammalian Cells (Miller and Calos, eds., Cold Spring Harbor Laboratory, N.Y., p. 10, (1987); all of which are herein incorporated by reference in their entirety).

The invention now being generally described, it will be more readily understood by reference to the following examples which are included for purposes of illustration only and are not intended to limit the present invention.

EXAMPLES

Example 1: Identification of Prenyltransferase Sequences

PSI-BLAST (Altschul, *et al.* (1997) *Nuc Acid Res* 25:3389-3402) profiles were generated for both the straight chain and aromatic classes of prenyltransferases. To generate the straight chain profile, a prenyl- transferase from *Porphyra purpurea* (Genbank accession 1709766) was used as a query against the NCBI non-redundant protein database. The *E. coli* enzyme involved in the formation of ubiquinone, ubiA (genbank accession 1790473) was used as a starting sequence to generate the aromatic prenyltransferase profile. These profiles were used to search public and proprietary DNA and protein data bases. In *Arabidopsis* seven putative prenyltransferases of the straight-chain class were identified, ATPT1, (SEQ ID NO:9), ATPT7 (SEQ ID NO:10), ATPT8 (SEQ ID NO:11), ATPT9 (SEQ ID NO:13), ATPT10 (SEQ ID NO:14), ATPT11 (SEQ ID NO:15), and ATPT12 (SEQ ID NO:16) and five were identified of the aromatic class, ATPT2 (SEQ ID NO:1), ATPT3 (SEQ ID NO:3), ATPT4 (SEQ ID NO:5), ATPT5 (SEQ ID NO:7), ATPT6 (SEQ ID NO:8). Additional prenyltransferase sequences from other plants related to the aromatic class of prenyltransferases, such as soy (SEQ ID NOs: 19-23, the deduced amino acid sequence of SEQ ID NO:23 is provided in SEQ ID NO:24) and maize (SEQ ID NOs:25-29, and 31) are also identified. The deduced amino acid sequence of ZMPT5 (SEQ ID NO:29) is provided in SEQ ID NO:30.

Searches are performed on a Silicon Graphics Unix computer using additional Bioaccellerator hardware and GenWeb software supplied by Compugen Ltd. This software and hardware enables the use of the Smith-Waterman algorithm in searching DNA and protein databases using profiles as queries. The program used to query protein databases is

profilesearch. This is a search where the query is not a single sequence but a profile based on a multiple alignment of amino acid or nucleic acid sequences. The profile is used to query a sequence data set, i.e., a sequence database. The profile contains all the pertinent information for scoring each position in a sequence, in effect replacing the "scoring matrix" used for the standard query searches. The program used to query nucleotide databases with a protein profile is tprofilesearch. Tprofilesearch searches nucleic acid databases using an amino acid profile query. As the search is running, sequences in the database are translated to amino acid sequences in six reading frames. The output file for tprofilesearch is identical to the output file for profilesearch except for an additional column that indicates the frame in which the best alignment occurred.

The Smith-Waterman algorithm, (Smith and Waterman (1981) *supra*), is used to search for similarities between one sequence from the query and a group of sequences contained in the database. E score values as well as other sequence information, such as conserved peptide sequences are used to identify related sequences.

To obtain the entire coding region corresponding to the *Arabidopsis* prenyltransferase sequences, synthetic oligo-nucleotide primers are designed to amplify the 5' and 3' ends of partial cDNA clones containing prenyltransferase sequences. Primers are designed according to the respective *Arabidopsis* prenyltransferase sequences and used in Rapid Amplification of cDNA Ends (RACE) reactions (Frohman *et al.* (1988) *Proc. Natl. Acad. Sci. USA* 85:8998-9002) using the Marathon cDNA amplification kit (Clontech Laboratories Inc, Palo Alto, CA).

Additional BLAST searches are performed using the ATPT2 sequence, a sequence in the class of aromatic prenyl transferases. Additional sequences are identified in soybean libraries that are similar to the ATPT2 sequence. The additional soybean sequence demonstrates 80% identity and 91% similarity at the amino acid sequence.

Amino acid sequence alignments between ATPT2 (SEQ ID NO:2), ATPT3 (SEQ ID NO:4), ATPT4 (SEQ ID NO:6), ATPT8 (SEQ ID NO:12), and ATPT12 (SEQ ID NO:17) are performed using ClustalW (Figure 1), and the percent identity and similarities are provided in Table 1 below.

Table 1:

	ATPT2	ATPT3	ATPT4	ATPT8	ATPT12

ATPT2 % Identity		12	13	11	15
% similar		25	25	22	32
% Gap		17	20	20	9
ATPT3 % Identity			12	6	22
% similar			29	16	38
% Gap			20	24	14
ATPT4 % Identity				9	14
% similar				18	29
% Gap				26	19
ATPT8 % Identity					7
% similar					19
% Gap					20
ATPT12 % Identity					
% similar					
% Gap					

Example 2: Preparation of Expression Constructs

A plasmid containing the napin cassette derived from pCGN3223 (described in USPN 5,639,790, the entirety of which is incorporated herein by reference) was modified to make it more useful for cloning large DNA fragments containing multiple restriction sites, and to allow the cloning of multiple napin fusion genes into plant binary transformation vectors. An adapter comprised of the self annealed oligonucleotide of sequence

CGCGATTTAAATGGCGCGCCCTGCAGGCGGCCGCCTGCAGGGCGCGCCATTTAA AT (SEQ ID NO:40) was ligated into the cloning vector pBC SK+ (Stratagene) after digestion with the restriction endonuclease BssHII to construct vector pCGN7765. Plamids pCGN3223 and pCGN7765 were digested with NotI and ligated together. The resultant vector, pCGN7770, contains the pCGN7765 backbone with the napin seed specific expression cassette from pCGN3223.

The cloning cassette, pCGN7787, essentially the same regulatory elements as pCGN7770, with the exception of the napin regulatory regions of pCGN7770 have been

replaced with the double CAMV 35S promoter and the tml polyadenylation and transcriptional termination region.

A binary vector for plant transformation, pCGN5139, was constructed from pCGN1558 (McBride and Summerfelt, (1990) Plant Molecular Biology, 14:269-276). The polylinker of pCGN1558 was replaced as a HindIII/Asp718 fragment with apolylinker containing unique restriction endonuclease sites, AscI, PacI, XbaI, SwaI, BamHI, and NotI. The Asp718 and HindIII restriction endonuclease sites are retained in pCGN5139.

A series of turbo binary vectors are constructed to allow for the rapid cloning of DNA sequences into binary vectors containing transcriptional initiation regions (promoters) and transcriptional termination regions.

The plasmid pCGN8618 was constructed by ligating oligonucleotides 5'-TCGAGGATCCGCGGCCGCAAGCTTCCTGCAGG-3' (SEQ ID NO:41) and 5'-TCGACCTGCAGGAAGCTTGCGGCCGCGGATCC-3' (SEQ ID NO:42) into SalI/XhoI-digested pCGN7770. A fragment containing the napin promoter, polylinker and napin 3' region was excised from pCGN8618 by digestion with Asp718I; the fragment was blunt-ended by filling in the 5' overhangs with Klenow fragment then ligated into pCGN5139 that had been digested with Asp718I and HindIII and blunt-ended by filling in the 5' overhangs with Klenow fragment. A plasmid containing the insert oriented so that the napin promoter was closest to the blunted Asp718I site of pCGN5139 and the napin 3' was closest to the blunted HindIII site was subjected to sequence analysis to confirm both the insert orientation and the integrity of cloning junctions. The resulting plasmid was designated pCGN8622.

The plasmid pCGN8619 was constructed by ligating oligonucleotides 5'-TCGACCTGCAGGAAGCTTGCGGCCGCGGATCC -3' (SEQ ID NO:43) and 5'-TCGAGGATCCGCGGCCGCAAGCTTCCTGCAGG-3' (SEQ ID NO:44) into SalI/XhoI-digested pCGN7770. A fragment containing the napin promoter, polylinker and napin 3' region was removed from pCGN8619 by digestion with Asp718I; the fragment was blunt-ended by filling in the 5' overhangs with Klenow fragment then ligated into pCGN5139 that had been digested with Asp718I and HindIII and blunt-ended by filling in the 5' overhangs with Klenow fragment. A plasmid containing the insert oriented so that the napin promoter was closest to the blunted Asp718I site of pCGN5139 and the napin 3' was closest to the blunted HindIII site was subjected to sequence analysis to confirm both the insert orientation and the integrity of cloning junctions. The resulting plasmid was designated pCGN8623.

The plasmid pCGN8620 was constructed by ligating oligonucleotides 5'-TCGAGGATCCGCGGCCGCAAGCTTCCTGCAGGAGCT -3' (SEQ ID NO:45) and 5'-CCTGCAGGAAGCTTGCGGCCGCGGATCC-3' (SEQ ID NO:46) into SalI/SacI-digested pCGN7787. A fragment containing the d35S promoter, polylinker and tml 3' region was removed from pCGN8620 by complete digestion with Asp718I and partial digestion with NotI. The fragment was blunt-ended by filling in the 5' overhangs with Klenow fragment then ligated into pCGN5139 that had been digested with Asp718I and HindIII and blunt-ended by filling in the 5' overhangs with Klenow fragment. A plasmid containing the insert oriented so that the d35S promoter was closest to the blunted Asp718I site of pCGN5139 and the tml 3' was closest to the blunted HindIII site was subjected to sequence analysis to confirm both the insert orientation and the integrity of cloning junctions. The resulting plasmid was designated pCGN8624.

The plasmid pCGN8621 was constructed by ligating oligonucleotides 5'-TCGACCTGCAGGAAGCTTGCGGCCGCGGATCCAGCT -3' (SEQ ID NO:47) and 5'-GGATCCGCGGCCGCAAGCTTCCTGCAGG-3' (SEQ ID NO:48) into SalI/SacI-digested pCGN7787. A fragment containing the d35S promoter, polylinker and tml 3' region was removed from pCGN8621 by complete digestion with Asp718I and partial digestion with NotI. The fragment was blunt-ended by filling in the 5' overhangs with Klenow fragment then ligated into pCGN5139 that had been digested with Asp718I and HindIII and blunt-ended by filling in the 5' overhangs with Klenow fragment. A plasmid containing the insert oriented so that the d35S promoter was closest to the blunted Asp718I site of pCGN5139 and the tml 3' was closest to the blunted HindIII site was subjected to sequence analysis to confirm both the insert orientation and the integrity of cloning junctions. The resulting plasmid was designated pCGN8625.

The plasmid construct pCGN8640 is a modification of pCGN8624 described above. A 938bp PstI fragment isolated from transposon Tn7 which encodes bacterial spectinomycin and streptomycin resistance (Fling et al. (1985), *Nucleic Acids Research* 13(19):7095-7106), a determinant for E. coli and Agrobacterium selection, was blunt ended with Pfu polymerase. The blunt ended fragment was ligated into pCGN8624 that had been digested with SpeI and blunt ended with Pfu polymerase. The region containing the PstI fragment was sequenced to confirm both the insert orientation and the integrity of cloning junctions.

The spectinomycin resistance marker was introduced into pCGN8622 and pCGN8623 as follows. A 7.7 Kbp AvrII-SnaBI fragment from pCGN8640 was ligated to a 10.9 Kbp

AvrII-SnaBI fragment from pCGN8623 or pCGN8622, described above. The resulting plasmids were pCGN8641 and pCGN8643, respectively.

The plasmid pCGN8644 was constructed by ligating oligonucleotides 5'-GATCACCTGCAGGAAGCTTGCGGCCGCGGATCCAATGCA-3' (SEQ ID NO:49) and 5'- TTGGATCCGCGGCCGCAAGCTTCCTGCAGGT-3' (SEQ ID NO:50) into BamHI-PstI digested pCGN8640.

Synthetic oligonulceotides were designed for use in Polymerase Chain Reactions (PCR) to amplify the coding sequences of ATPT2, ATPT3, ATPT4, ATPT8, and ATPT12 for the preparation of expression constructs and are provided in Table 2 below.

Table 2:

Name	Restriction Site	Sequence	SEQ ID NO:
ATPT2	5' NotI	GGATCCGCGGCCGCACAATGGAGTC TCTGCTCTCTAGTTCT	51
ATPT2	3' SseI	GGATCCTGCAGGTCACTTCAAAAAA GGTAACAGCAAGT	52
ATPT3	5' NotI	GGATCCGCGGCCGCACAATGGCGTT TTTTGGGCTCTCCCGTGTTT	53
ATPT3	3' SseI	GGATCCTGCAGGTTATTGAAAACTT CTTCCAAGTACAACT	54
ATPT4	5' NotI	GGATCCGCGGCCGCACAATGTGGCG AAGATCTGTTGTT	55
ATPT4	3' SseI	GGATCCTGCAGGTCATGGAGAGTAG AAGGAAGGAGCT	56
ATPT8	5' NotI	GGATCCGCGGCCGCACAATGGTACT TGCCGAGGTTCCAAAGCTTGCCTCT	57
ATPT8	3' SseI	GGATCCTGCAGGTCACTTGTTTCTG GTGATGACTCTAT	58
ATPT12	5' NotI	GGATCCGCGGCCGCACAATGACTTC GATTCTCAACACT	59
ATPT12	3' SseI	GGATCCTGCAGGTCAGTGTTGCGAT GCTAATGCCGT	60

The coding sequences of ATPT2, ATPT3, ATPT4, ATPT8, and ATPT12 were all amplified using the respective PCR primers shown in Table 2 above and cloned into the TopoTA vector (Invitrogen). Constructs containing the respective prenyltransferase sequences were digested with NotI and Sse8387I and cloned into the turbobinary vectors described above.

The sequence encoding ATPT2 prenyltransferase was cloned in the sense orientation into pCGN8640 to produce the plant transformation construct pCGN10800 (Figure 2). The ATPT2 sequence is under control of the 35S promoter.

The ATPT2 sequence was also cloned in the antisense orientation into the construct pCGN8641 to create pCGN10801 (Figure 3). This construct provides for the antisense expression of the ATPT2 sequence from the napin promoter.

The ATPT2 coding sequence was also cloned in the antisense orientation into the vector pCGN8643 to create the plant transformation construct pCGN10802

The ATPT2 coding sequence was also cloned in the antisense orientation into the vector pCGN8644 to create the plant transformation construct pCGN10803 (Figure 4).

The ATPT4 coding sequence was cloned into the vector pCGN864 to create the plant transformation construct pCGN10806 (Figure 5). The ATPT2 coding sequence was cloned into the vector pCGN864 to create the plant transformation construct pCGN10807(Figure 6). The ATPT3 coding sequence was cloned into the vector pCGN864 to create the plant transformation construct pCGN10808 (Figure 7). The ATPT3 coding sequence was cloned in the sense orientation into the vector pCGN8640 to create the plant transformation construct pCGN10809 (Figure 8). The ATPT3 coding sequence was cloned in the antisense orientation into the vector pCGN8641 to create the plant transformation construct pCGN10810 (Figure 9). The ATPT3 coding sequence was cloned into the vector pCGN8643 to create the plant transformation construct pCGN10811 (Figure 10). The ATPT3 coding sequence was cloned into the vector pCGN8640 to create the plant transformation construct pCGN10812 (Figure 11). The ATPT4 coding sequence was cloned into the vector pCGN8640 to create the plant transformation construct pCGN10813 (Figure 12). The ATPT4 coding sequence was cloned into the vector pCGN8643 to create the plant transformation construct pCGN10814 (Figure 13). The ATPT4 coding sequence was cloned into the vector pCGN8641 to create the plant transformation construct pCGN10815 (Figure 14). The ATPT4 coding sequence was cloned in the antisense orientation into the vector pCGN8644 to create the plant transformation construct pCGN10816 (Figure 15). The ATPT2 coding sequence was cloned into the vector pCGN???? to create the plant transformation construct pCGN10817 (Figure 16). The ATPT8 coding sequence was cloned in the sense orientation into the vector pCGN8643 to create the plant transformation construct pCGN10819 (Figure 17). The ATPT12 coding sequence was cloned into the vector pCGN8644 to create the plant transformation construct pCGN10824 (Figure 18). The ATPT12 coding sequence was cloned into the vector pCGN8641 to create the plant transformation construct pCGN10825 (Figure 19). The ATPT8 coding sequence was cloned into the vector pCGN8644 to create the plant transformation construct pCGN10826 (Figure 20).

Example 3: Plant Transformation

Transgenic *Brassica* plants are obtained by *Agrobacterium*-mediated transformation as described by Radke *et al.* (*Theor. Appl. Genet.* (1988) *75*:685-694; *Plant Cell Reports* (1992) *11*:499-505). Transgenic *Arabidopsis thaliana* plants may be obtained by *Agrobacterium*-mediated transformation as described by Valverkens *et al.*, (*Proc. Nat. Acad. Sci.* (1988) *85*:5536-5540), or as described by Bent et al. ((1994), *Science* 265:1856-1860), or Bechtold et al. ((1993), *C.R.Acad.Sci, Life Sciences* 316:1194-1199). Other plant species may be similarly transformed using related techniques.

Alternatively, microprojectile bombardment methods, such as described by Klein *et al.* (*Bio/Technology 10*:286-291) may also be used to obtain nuclear transformed plants.

Example 4: Identification of Additional Prenyltransferases

A PSI-Blast profile generated using the *E. coli* ubiA (genbank accession 1790473) sequence was used to analyze the *Synechocystis* genome. This analysis identified 5 open reading frames (ORFs) in the *Synechocystis* genome that were potentially prenyltransferases; slr0926 (annotated as ubiA (4-hydroxybenzoate-octaprenyl transferase, SEQ ID NO:32), sll1899 (annotated as ctaB (cytocrome c oxidase folding protein, SEQ ID NO:33), slr0056 (annotated as g4 (chlorophyll synthase 33 kd subunit, SEQ ID NO:34), slr1518 (annotated as menA (menaquinone biosynthesis protein, SEQ ID NO:35), and slr1736 (annotated as a hypothetical protein of unknown function (SEQ ID NO:36).

To determine the functionality of these ORFs and their involvement, if any, in the biosynthesis of Tocopherols, knockouts constructs were made to disrupt the ORF identified in *Synechocystis*.

Synthetic oligos were designed to amplify regions from the 5' (5'-TAATGTGTACATTGTCGGCCTC (17365') (SEQ ID NO:61) and 5'-GCAATGTAACATCAGAGATTTTGAGACACAACGTGGCTTTCCACAATTCCCCGCACCGTC (1736kanpr1)) (SEQ ID NO:62) and 3' (5'-AGGCTAATAAGCACAAATGGGA (17363') (SEQ ID NO:63) and 5'-

GGTATGAGTCAGCAACACCTTCTTCACGAGGCAGACCTCAGC GGAATTGGTTTAGGTTATCCC (1736kanpr2)) (SEQ ID NO:64) ends of the slr1736 ORF. The 1736kanpr1 and 1736kanpr2 oligos contained 20 bp of homology to the slr1736 ORF with an additional 40 bp of sequence homology to the ends of the kanamycin resistance cassette. Separate PCR steps were completed with these oligos and the products were gel purified and combined with the kanamycin resistance gene from puc4K (Pharmacia) that had been digested with *Hinc*II and gel purified away from the vector backbone. The combined fragments were allowed to assemble without oligos under the following conditions: 94°C for 1 min, 55°C for 1 min, 72°C for 1 min plus 5 seconds per cycle for 40 cycles using pfu polymerase in 100ul reaction volume (Zhao, H and Arnold (1997) *Nucleic Acids Res.* 25(6):1307-1308). One microliter or five microliters of this assembly reaction was then amplified using 5' and 3' oligos nested within the ends of the ORF fragment, so that the resulting product contained 100-200 bp of the 5' end of the *Synechocystis* gene to be knocked out, the kanamycin resistance cassette, and 100-200 bp of the 3' end of the gene to be knocked out. This PCR product was then cloned into the vector pGemT easy (Promega) to create the construct pMON21681 and used for *Synechocystis* transformation.

Primers were also synthesized for the preparation of *Synechocystis* knockout constructs for the other sequences using the same method as described above, with the following primers. The ubiA 5' sequence was amplified using the primers 5'-GGATCCATGGTT GCCCAAACCCCATC (SEQ ID NO:65) and 5'-GCAATGTAACATCAGAGA TTTTGAGACACAACG TGGCTTTGGGTAAGCAACAATGACCGGC (SEQ ID NO:66). The 3' region was amplified using the synthetic oligonucleotide primers 5'-GAATTCTCAAAGCCAGCCCAGTAAC (SEQ ID NO:67) and 5'-GGTATGAGTC AGCAACACCTTCTTCACGAGGCAGACCTCAGCGGGTGCGAAAAGGGTTTTCCC (SEQ ID NO:68). The amplification products were combined with the kanamycin resistance gene from puc4K (Pharmacia) that had been digested with *Hinc*II and gel purified away from the vector backbone. The annealed fragment was amplified using 5' and 3' oligos nested within the ends of the ORF fragment (5'- CCAGTGGTTTAGGCTGTGTGGTC (SEQ ID NO:69) and 5'- CTGAGTTGGATGTATTGGATC (SEQ ID NO:70)), so that the resulting product contained 100-200 bp of the 5' end of the *Synechocystis* gene to be knocked out, the kanamycin resistance cassette, and 100-200 bp of the 3' end of the gene to be knocked out.

This PCR product was then cloned into the vector pGemT easy (Promega) to create the construct pMON21682 and used for *Synechocystis* transformation.

Primers were also synthesized for the preparation of *Synechocystis* knockout constructs for the other sequences using the same method as described above, with the following primers. The sl11899 5' sequence was amplified using the primers 5'-GGATCCATGGTTACTT CGACAAAAATCC (SEQ ID NO:71) and 5'-GCAATGTAACATCAGAG ATTTTGAGACACAACGTGGCTTTGCTAGGCAACCGCTTAGTAC (SEQ ID NO:72). The 3' region was amplified using the synthetic oligonucleotide primers 5'-GAATTCTTAACCCAACAGTAAAGTTCCC (SEQ ID NO:73) and 5'-GGTATGAGTCAGC AACACCTTCTTCACGAGGCAGACCTCAGCGCCGGCATTGTCTTTTACATG (SEQ ID NO:74). The amplification products were combined with the kanamycin resistance gene from puc4K (Pharmacia) that had been digested with *Hinc*II and gel purified away from the vector backbone. The annealed fragment was amplified using 5' and 3' oligos nested within the ends of the ORF fragment (5'- GGAACCCTTGCAGCCGCTTC (SEQ ID NO:75) and 5'- GTATGCCCAACTGGTGCAGAGG (SEQ ID NO:76)), so that the resulting product contained 100-200 bp of the 5' end of the *Synechocystis* gene to be knocked out, the kanamycin resistance cassette, and 100-200 bp of the 3' end of the gene to be knocked out. This PCR product was then cloned into the vector pGemT easy (Promega) to create the construct pMON21679 and used for *Synechocystis* transformation.

Primers were also synthesized for the preparation of *Synechocystis* knockout constructs for the other sequences using the same method as described above, with the following primers. The slr0056 5' sequence was amplified using the primers 5'-GGATCCATGTCTGACACACAAAATACCG (SEQ ID NO:77) and 5'-GCAATGTAACATCAGAGATTTTGAGACACAACGTGGCTTTCGCCAATACCAGCCA CCAACAG (SEQ ID NO:78). The 3' region was amplified using the synthetic oligonucleotide primers 5'- GAATTCTCAAAT CCCCGCATGGCCTAG (SEQ ID NO:79) and 5'-GGTATGAGTCAGCAACACCTTCTTCACGAGGCAGACCTCAGCGGCCTACGGCTTG GACGTGTGGG (SEQ ID NO:80). The amplification products were combined with the kanamycin resistance gene from puc4K (Pharmacia) that had been digested with *Hinc*II and gel purified away from the vector backbone. The annealed fragment was amplified using 5'

and 3' oligos nested within the ends of the ORF fragment (5'-CACTTGGATTCCCCTGATCTG (SEQ ID NO:81) and 5'-GCAATACCCGCTTGGAAAACG (SEQ ID NO:82)), so that the resulting product contained 100-200 bp of the 5' end of the *Synechocystis* gene to be knocked out, the kanamycin resistance cassette, and 100-200 bp of the 3' end of the gene to be knocked out. This PCR product was then cloned into the vector pGemT easy (Promega) to create the construct pMON21677 and used for *Synechocystis* transformation.

Primers were also synthesized for the preparation of *Synechocystis* knockout constructs for the other sequences using the same method as described above, with the following primers. The slr1518 5' sequence was amplified using the primers 5'-GGATCCATGACCGAAT CTTCGCCCCTAGC (SEQ ID NO:83) and 5'-GCAATGTAACATCAGAGATTTTGA GACACAACGTGGC TTTCAATCCTAGGTAGCCGAGGCG (SEQ ID NO:84). The 3' region was amplified using the synthetic oligonucleotide primers 5'- GAATTCTTAGCCCAGGCC AGCCCAGCC (SEQ ID NO:85)and 5'- GGTATGAGTCAGCAACACCTTCTTCACGA GGCAGACCTCAGCGGGGAATTGATTTGTTTAATTACC (SEQ ID NO:86). The amplification products were combined with the kanamycin resistance gene from puc4K (Pharmacia) that had been digested with *Hinc*II and gel purified away from the vector backbone. The annealed fragment was amplified using 5' and 3' oligos nested within the ends of the ORF fragment (5'- GCGATCGCCATTATCGCTTGG (SEQ ID NO:87) and 5'-GCAGACTGGCAATTATCAGTAACG (SEQ ID NO:88)), so that the resulting product contained 100-200 bp of the 5' end of the *Synechocystis* gene to be knocked out, the kanamycin resistance cassette, and 100-200 bp of the 3' end of the gene to be knocked out. This PCR product was then cloned into the vector pGemT easy (Promega) to create the construct pMON21680 and used for *Synechocystis* transformation.

B. Transformation of *Synechocystis*

Cells of *Synechocystis* 6803 were grown to a density of approximately $2x10^8$ cells per ml and harvested by centrifugation. The cell pellet was re-suspended in fresh BG-11 medium (ATCC Medium 616) at a density of $1x10^9$ cells per ml and used immediately for transformation. One-hundred microliters of these cells were mixed with 5 ul of mini prep DNA and incubated with light at 30C for 4 hours. This mixture was then plated onto nylon filters resting on BG-11 agar supplemented with TES pH8 and allowed to grow for 12-18

hours. The filters were then transferred to BG-11 agar + TES + 5ug/ml kanamycin and allowed to grow until colonies appeared within 7-10 days (Packer and Glazer, 1988). Colonies were then picked into BG-11 liquid media containing 5 ug/ml kanamycin and allowed to grow for 5 days. These cells were then transferred to Bg-11 media containing 10ug/ml kanamycin and allowed to grow for 5 days and then transferred to Bg-11 + kanamycin at 25ug/ml and allowed to grow for 5 days. Cells were then harvested for PCR analysis to determine the presence of a disrupted ORF and also for HPLC analysis to determine if the disruption had any effect on tocopherol levels.

PCR analysis of the *Synechocystis* isolates for slr1736 and sll1899 showed complete segregation of the mutant genome, meaning no copies of the wild type genome could be detected in these strains. This suggests that function of the native gene is not essential for cell function. HPLC analysis of these same isolates showed that the sll1899 strain had no detectable reduction in tocopherol levels. However, the strain carrying the knockout for slr1736 produced no detectable levels of tocopherol.

The amino acid sequences for the *Synechocystis* knockouts are compared using ClustalW, and are provided in Table 3 below. Provided are the percent identities, percent similarity, and the percent gap. The alignment of the sequences is provided in Figure 21.

Table 3:

	Slr1736	slr0926	sll1899	slr0056	slr1518
slr1736 %identity		14	12	18	11
%similar		29	30	34	26
%gap		8	7	10	5
slr0926 %identity			20	19	14
%similar			39	32	28
%gap			7	9	4
sll1899 %identity				17	13
%similar				29	29
%gap				12	9
slr0056 %identity					15
%similar					31
%gap					8

slr1518 %identity %similar %gap	

Amino acid sequence comparisons are performed using various *Arabidopsis* prenyltransferase sequences and the *Synechocystis* sequences. The comparisons are presented in Table 4 below. Provided are the percent identities, percent similarity, and the percent gap. The alignment of the sequences is provided in Figure 22.

Table 4:

	ATPT2	slr1736	ATPT3	slr0926	ATPT4	sll1899	ATPT12	slr0056	ATPT8	slr1518
ATPT2		29	9	9	8	8	12	9	7	9
		46	23	21	20	20	28	23	21	20
		27	13	28	23	29	11	24	25	24
slr1736			9	13	8	12	13	15	8	10
			19	28	19	28	26	33	21	26
			34	12	34	15	26	10	12	10
ATPT3				23	11	14	13	10	5	11
				36	26	26	26	21	14	22
				29	21	31	16	30	30	30
					12	20	17	20	11	14
slr0926					24	37	28	33	24	29
					33	12	25	10	11	9
						18	11	8	6	7
ATPT4						33	23	18	16	19
						28	19	32	32	33
							13	17	10	12
sll1899							24	30	23	26
							27	13	10	11
								52	8	11
ATPT12								66	19	26
								18	25	23

		9	13
slr0056		23	32
		10	8
			7
ATPT8			23
			7
slr1518			

4B. Preparation of the slr1737 Knockout

The *Synechocystis sp. 6803* slr1737 knockout was constructed by the following method. The GPS™-1 Genome Priming System (New England Biolabs) was used to insert, by a Tn7 Transposase system, a Kanamycin resistance cassette into *slr1737*. A plasmid from a *Synechocystis* genomic library clone containing 652 base pairs of the targeted orf (*Synechcocystis* genome base pairs 1324051 – 1324703; the predicted orf base pairs 1323672 – 1324763, as annotated by Cyanobase) was used as target DNA. The reaction was performed according to the manufacturers protocol. The reaction mixture was then transformed into *E. coli* DH10B electrocompetant cells and plated. Colonies from this transformation were then screened for transposon insertions into the target sequence by amplifying with M13 Forward and Reverse Universal primers, yielding a product of 652 base pairs plus ~1700 base pairs, the size of the transposon kanamycin cassette, for a total fragment size of ~2300 base pairs. After this determination, it was then necessary to determine the approximate location of the insertion within the targeted orf, as 100 base pairs of orf sequence was estimated as necessary for efficient homologous recombination in *Synechocystis*. This was accomplished through amplification reactions using either of the primers to the ends of the transposon, Primer S (5' end) or N (3' end), in combination with either a M13 Forward or Reverse primer. That is, four different primer combinations were used to map each potential knockout construct: Primer S – M13 Forward, Primer S – M13 Reverse, Primer N – M13 Forward, Primer N – M13 Reverse. The construct used to transform *Synechocystis* and knockout slr1737 was determined to consist of a approximately

150 base pairs of slr1737 sequence on the 5' side of the transposon insertion and approximately 500 base pairs on the 3' side, with the transcription of the orf and kanamycin cassette in the same direction. The nucleic acid sequence of slr1737 is provided in SEQ ID NO:38 the deduced amino acid sequence is provided in SEQ ID NO:39.

Cells of *Synechocystis 6803* were grown to a density of ~ $2x10^8$ cells per ml and harvested by centrifugation. The cell pellet was re-suspended in fresh BG-11 medium at a density of $1x10^9$ cells per ml and used immediately for transformation. 100 ul of these cells were mixed with 5 ul of mini prep DNA and incubated with light at 30C for 4 hours. This mixture was then plated onto nylon filters resting on BG-11 agar supplemented with TES ph8 and allowed to grow for 12-18 hours. The filters were then transferred to BG-11 agar + TES + 5ug/ml kanamycin and allowed to grow until colonies appeared within 7-10 days (Packer and Glazer, 1988). Colonies were then picked into BG-11 liquid media containing 5 ug/ml kanamycin and allowed to grow for 5 days. These cells were then transferred to Bg-11 media containing 10ug/ml kanamycin and allowed to grow for 5 days and then transferred to Bg-11 + kanamycin at 25ug/ml and allowed to grow for 5 days. Cells were then harvested for PCR analysis to determine the presence of a disrupted ORF and also for HPLC analysis to determine if the disruption had any effect on tocopherol levels.

PCR analysis of the *Synechocystis* isolates, using primers to the ends of the *slr1737* orf , showed complete segregation of the mutant genome, meaning no copies of the wild type genome could be detected in these strains. This suggests that function of the native gene is not essential for cell function. HPLC analysis of the strain carrying the knockout for *slr1737* produced no detectable levels of tocopherol.

4C. Phytyl Prenyltransferase Enzyme Assays

[^{3}H] Homogentisic acid in 0.1% H_3PO_4 (specific radioactivity 40 Ci/mmol). Phytyl pyrophosphate was synthesized as described by Joo, *et al.* (1973) *Can J. Biochem.* 51:1527. 2-methyl-6-phytylquinol and 2,3-dimethyl-5-phytylquinol were synthesized as described by Soll, *et al.* (1980) *Phytochemistry* 19:215. Homogentisic acid, α, β, δ, and γ-tocopherol, and tocol, were purchased commercially.

The wild-type strain of *Synechocystis* sp. PCC 6803 was grown in BG11 medium with bubbling air at 30°C under 50 $\mu E.m^{-2}.s^{-1}$ fluorescent light, and 70% relative humidity. The growth medium of slr1736 knock-out (potential PPT) strain of this organism was

supplemented with 25 μg mL^{-1} kanamycin. Cells were collected from 0.25 to 1 liter culture by centrifugation at 5000 *g* for 10 min and stored at -80°C.

Total membranes were isolated according to Zak's procedures with some modifications (Zak, *et al.* (1999) *Eur J. Biochem* 261:311). Cells were broken on a French press. Before the French press treatment, the cells were incubated for 1 hour with lysozyme (0.5%, w/v) at 30 °C in a medium containing 7 mM EDTA, 5 mM NaCl and 10 mM Hepes-NaOH, pH 7.4. The spheroplasts were collected by centrifugation at 5000 *g* for 10 min and resuspended at 0.1 - 0.5 mg chlorophyll·mL^{-1} in 20 mM potassium phosphate buffer, pH 7.8. Proper amount of protease inhibitor cocktail and DNAase I from Boehringer Mannheim were added to the solution. French press treatments were performed two to three times at 100 MPa. After breakage, the cell suspension was centrifuged for 10 min at 5000g to pellet unbroken cells, and this was followed by centrifugation at 100 000 *g* for 1 hour to collect total membranes. The final pellet was resuspended in a buffer containing 50 mM Tris-HCL and 4 mM $MgCl_2$.

Chloroplast pellets were isolated from 250 g of spinach leaves obtained from local markets. Devined leaf sections were cut into grinding buffer (2 l /250 g leaves) containing 2 mM EDTA, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 0.33 M sorbitol, 0.1% ascorbic acid, and 50 mM Hepes at pH 7.5. The leaves were homogenized for 3 sec three times in a 1-L blendor, and filtered through 4 layers of mirocloth. The supernatant was then centrifuged at 5000*g* for 6 min. The chloroplast pellets were resuspended in small amount of grinding buffer (Douce,*et al* Methods in Chloroplast Molecular Biology, 239 (1982)

Chloroplasts in pellets can be broken in three ways. Chloroplast pellets were first aliquoted in 1 mg of chlorophyll per tube, centrifuged at 6000 rpm for 2 min in microcentrifuge, and grinding buffer was removed. Two hundred microliters of Triton X-100 buffer (0.1% Triton X-100, 50 mM Tris-HCl pH 7.6 and 4 mM $MgCl_2$) or swelling buffer (10 mM Tris pH 7.6 and 4 mM $MgCl_2$) was added to each tube and incubated for ½ hour at 4°C. Then the broken chloroplast pellets were used for the assay immediately. In addition, broken chloroplasts can also be obtained by freezing in liquid nitrogen and stored at -80°C for ½ hour, then used for the assay.

In some cases chloroplast pellets were further purified with 40%/ 80% percoll gradient to obtain intact chloroplasts. The intact chloroplasts were broken with swelling buffer, then either used for assay or further purified for envelope membranes with 20.5%/ 31.8% sucrose density gradient (Sol, *et al* (1980) *supra*). The membrane fractions were centrifuged at 100 000g for 40 min and resuspended in 50 mM Tris-HCl pH 7.6, 4 mM $MgCl_2$.

Various amounts of [^{3}H]HGA, 40 to 60 μM unlabelled HGA with specific activity in the range of 0.16 to 4 Ci/mmole were mixed with a proper amount of 1M Tris-NaOH pH 10 to adjust pH to 7.6. HGA was reduced for 4 min with a trace amount of solid $NaBH_4$. In addition to HGA, standard incubation mixture (final vol 1 mL) contained 50 mM Tris-HCl, pH 7.6, 3-5 mM $MgCl_2$, and 100 μM phytyl pyrophosphate. The reaction was initiated by addition of *Synechocystis* total membranes, spinach chloroplast pellets, spinach broken chloroplasts, or spinach envelope membranes. The enzyme reaction was carried out for 2 hour at 23°C or 30°C in the dark or light. The reaction is stopped by freezing with liquid nitrogen, and stored at -80°C or directly by extraction.

A constant amount of tocol was added to each assay mixture and reaction products were extracted with a 2 mL mixture of chloroform/methanol (1:2, v/v) to give a monophasic solution. NaCl solution (2 mL; 0.9%) was added with vigorous shaking. This extraction procedure was repeated three times. The organic layer containing the prenylquinones was filtered through a 20 mμ filter, evaporated under N_2, and then resuspended in 100 μL of ethanol.

The samples were mainly analyzed by Normal-Phase HPLC method (Isocratic 90% Hexane and 10% Methyl-t-butyl ether), and use a Zorbax silica column, 4.6 x 250 mm. The samples were also analyzed by Reversed-Phase HPLC method (Isocratic 0.1% H_3PO_4 in MeOH), and use a Vydac 201HS54 C18 column; 4.6 x 250 mm coupled with an All-tech C18 guard column. The amount of products were calculated based on the substrate specific radioactivity, and adjusted according to the % recovery based on the amount of internal standard.

The amount of chlorophyll was determined as described in Arnon (1949) *Plant Physiol.* 24:1. Amount of protein was determined by the Bradford method using gamma globulin as a standard (Bradford, (1976) *Anal. Biochem.* 72:248)

Results of the assay demonstrate that 2-Methyl-6-Phytylplastoquinone is produced in the *Synechocystis* slr1736 knockout preparations. The results of the phytyl prenyltransferase enzyme activity assay for the slr1736 knock out are presented in Figure 23.

4D. Complementation of the slr1736 knockout with ATPT2

In order to determine whether ATPT2 could complement the knockout of slr1736 in *Synechocystis 6803* a plasmid was constructed to express the ATPT2 sequence from the TAC promoter. A vector, plasmid psl1211, was obtained from the lab of Dr. Himadri Pakrasi of

Washington University, and is based on the plasmid RSF1010 which is a broad host range plasmid (Ng W.-O., Zentella R., Wang, Y., Taylor J-S. A., Pakrasi, H.B. 2000. *phrA*, the major photoreactivating factor in the cyanobacterium *Synechocystis* sp. strain PCC 6803 codes for a cyclobutane pyrimidine dimer specific DNA photolyase. *Arch. Microbiol.* (in press)). The ATPT2 gene was isolated from the vector pCGN10817 by PCR using the following primers. ATPT2nco.pr 5'-CCATGGATTCGAGTAAAGTTGTCGC (SEQ ID NO:89); ATPT2ri.pr- 5'-GAATTCACTTCAAAAAAGGTAACAG (SEQ ID NO:90). These primers will remove approximately 112 BP from the 5' end of the ATPT2 sequence, which is thought to be the chloroplast transit peptide. These primers will also add an NcoI site at the 5' end and an EcoRI site at the 3' end which can be used for sub-cloning into subsequent vectors. The PCR product from using these primers and pCGN10817 was ligated into pGEM T easy and the resulting vector pMON21689 was confirmed by sequencing using the m13forward and m13reverse primers. The NcoI/EcoRI fragment from pMON21689 was then ligated with the EagI/EcoRI and EagI/NcoI fragments from psl1211 resulting in pMON21690. The plasmid pMON21690 was introduced into the slr1736 *Synechocystis 6803* KO strain via conjugation. Cells of sl906 (a helper strain) and DH10B cells containing pMON21690 were grown to log phase (O.D. 600= 0.4) and 1 ml was harvested by centrifugation. The cell pellets were washed twice with a sterile BG-11 solution and resuspended in 200 ul of BG-11. The following was mixed in a sterile eppendorf tube: 50 ul SL906, 50 ul DH10B cells containing pMON21690, and 100 ul of a fresh culture of the slr1736 *Synechocystis 6803* KO strain (O.D. 730 = 0.2-0.4). The cell mixture was immediately transferred to a nitrocellulose filter resting on BG-11 and incubated for 24 hours at 30C and 2500 LUX(50 ue) of light. The filter was then transferred to BG-11 supplemented with 10ug/ml Gentamycin and incubated as above for ~5 days. When colonies appeared, they were picked and grown up in liquid BG-11 + Gentamycin 10 ug/ml. (Elhai, J. and Wolk, P. 1988. Conjugal transfer of DNA to Cyanobacteria. *Methods in Enzymology* 167, 747-54) The liquid cultures were then assayed for tocopherols by harvesting 1ml of culture by centrifugation, extracting with ethanol/pyrogallol, and HPLC separation. The slr1736 *Synechocystis 6803* KO strain, did not contain any detectable tocopherols, while the slr1736 *Synechocystis 6803* KO strain transformed with pmon21690 contained detectable alpha tocopherol. A *Synechocystis 6803* strain transformed with psl1211(vector control) produced alpha tocopherol as well.

Example 5: Transgenic Plant Analysis

Arabidopsis plants transformed with constructs for the sense or antisense expression of the ATPT proteins were analyzed by High Pressure Liquid Chromatography (HPLC) for altered levels of total tocopherols, as well as altered levels of specific tocopherols (alpha, beta, gamma, and delta tocopherol).

Extracts of leaves and seeds were prepared for HPLC as follows. For seed extracts, 10 mg of seed was added to 1 g of microbeads (Biospec) in a sterile microfuge tube to which 500 ul 1% pyrogallol (Sigma Chem)/ethanol was added. The mixture was shaken for 3 minutes in a mini Beadbeater (Biospec) on "fast" speed. The extract was filtered through a 0.2 um filter into an autosampler tube. The filtered extracts were then used in HPLC analysis described below.

Leaf extracts were prepared by mixing 30-50 mg of leaf tissue with 1 g microbeads and freezing in liquid nitrogen until extraction. For extraction, 500 ul 1% pyrogallol in ethanol was added to the leaf/bead mixture and shaken for 1 minute on a Beadbeater (Biospec) on "fast" speed. The resulting mixture was centrifuged for 4 minutes at 14,000 rpm and filtered as described above prior to HPLC analysis.

HPLC was performed on a Zorbax silica HPLC column (4.6 mm X 250 mm) with a fluorescent detection, an excitation at 290 nm, an emission at 336 nm, and bandpass and slits. Solvent A was hexane and solvent B was methyl-t-butyl ether. The injection volume was 20 ul, the flow rate was 1.5 ml/min, the run time was 12 min (40°C) using the gradient (Table 5):

Table 5:

Time	Solvent A	Solvent B
0 min.	90%	10%
10 min.	90%	10%
11 min.	25%	75%
12 min.	90%	10%

Tocopherol standards in 1% pyrogallol/ ethanol were also run for comparison (alpha tocopherol, gamma tocopherol, beta tocopherol, delta tocopherol, and tocopherol (tocol) (all from Matreya).

Standard curves for alpha, beta, delta, and gamma tocopherol were calculated using Chemstation software. The absolute amount of component x is: Absolute amount of x=

Response$_x$ x RF$_x$ x dilution factor where Response$_x$ is the area of peak x, RF$_x$ is the response factor for component x (Amount$_x$/Response$_x$) and the dilution factor is 500 ul. The ng/mg tissue is found by: total ng component/mg plant tissue.

Results of the HPLC analysis of seed extracts of transgenic *Arabidopsis* lines containing pMON10822 for the expression of ATAT2 from the napin promoter are provided in Figure 24.

HPLC analysis results of *Arabidopsis* seed tissue expressing the ATAT2 sequence from the napin promoter (pMON10822) demonstrates an increased level of tocopherols in the seed. Total tocopherol levels are increased as much as 50 to 60% over the total tocopherol levels of non-transformed (wild-type) *Arabidopsis* plants (Figure 24).

Furthermore, increases of particular tocopherols are also increased in transgenic *Arabidopsis* plants expressing the ATAT2 nucleic acid sequence from the napin promoter. Levels of delta tocopherol in these lines are increased greater than 3 fold over the delta tocopherol levels obtained from the seeds of wild type *Arabidopsis* lines. Levels of gamma tocopherol in transgenic *Arabidopsis* lines expressing the ATAT2 nucleic acid sequence are increased as much as about 60% over the levels obtained in the seeds of non-transgenic control lines. Furthermore, levels of alpha tocopherol are increased as much as 3 fold over those obtained from non-transgenic control lines.

Results of the HPLC analysis of seed extracts of transgenic *Arabidopsis* lines containing pMON10803 for the expression of ATAT2 from the enhanced 35S promoter are provided in Figure 25.

All publications and patent applications mentioned in this specification are indicative of the level of skill of those skilled in the art to which this invention pertains. All publications and patent applications are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be obvious that certain changes and modifications may be practiced within the scope of the appended claim.

Claims

What is Claimed is:

1. An isolated nucleic acid sequence encoding a prenyltransferase.

2. An isolated nucleic acid sequence according to Claim 1, wherein said prenyltransferase is selected from the group consisting of straight chain prenyltransferase and aromatic prenyltransferase.

3. An isolated DNA sequence according to Claim 1, wherein said nucleic acid sequence is isolated from a eukaryotic cell source.

4. An isolated DNA sequence according to Claim 3, wherein said eukaryotic cell source is selected from the group consisting of mammalian, nematode, fungal, and plant cells.

5. The DNA encoding sequence of Claim 4 wherein said prenyltransferase protein is from *Arabidopsis*.

6. The DNA encoding sequence of Claim 5 wherein said prenyltransferase protein is encoded by a sequence selected from the group consisting of the sequences of Figure 1.

7. The DNA encoding sequence of Claim 4 wherein said prenyltransferase protein is from corn.

8. The DNA encoding sequence of Claim 7 wherein said prenyltransferase protein is encoded by a sequence which includes the EST of the sequences of Figure 3.

9. The DNA encoding sequence of Claim 4 wherein said prenyltransferase protein is from soybean.

10. The DNA encoding sequence of Claim 9 wherein said prenyltransferase protein is encoded by a sequence which includes the ESTs of the group consisting of the sequences of Figure 2 and Figure 9.

11. An isolated DNA sequence according to Claim 1, wherein said nucleic acid sequence is isolated from a prokaryotic cell source.

12. An isolated DNA sequence according to Claim 11, wherein said prokaryotic source is *Synechocystis*.

13. A nucleic acid construct comprising as operably linked components, a transcriptional initiation region functional in a host cell, a nucleic acid sequence encoding prenyltransferase, and a transcriptional termination region.

14. A nucleic acid construct according to Claim 13, wherein said nucleic acid sequence encoding prenyltransferase is obtained from an organism selected from the group consisting of a eukaryotic organism and a prokaryotic organism.

15. A nucleic acid construct according to Claim 14, wherein said nucleic acid sequence encoding prenyltransferase is obtained from a plant source.

16. A nucleic acid construct according to Claim 15, wherein said nucleic acid sequence encoding prenyltransferase is obtained from a source selected from the group consisting of *Arabidopsis,* soybean and corn.

17. A nucleic acid construct according to Claim 13, wherein said nucleic acid sequence encoding prenyltransferase is obtained from *Synechocystis.*

18. A plant cell comprising the construct of Claim13.

19. A method for the alteration of the tocopherol content in a host cell, comprising; transforming said host cell with a construct comprising as operably linked components, a transcriptional initiation region functional in a host cell, a nucleic acid sequence encoding prenyltransferase, and a transcriptional termination region.

20. The method according to Claim 19, wherein said host cell is selected from the group consisting of a prokaryotic cell and a eukaryotic cell.

21. The method according to Claim 20, wherein said prokaryotic cell is *Synechocystis.*

22. The method according to Claim 20, wherein said eukaryotic cell is a plant cell.

23. The method according to Claim 22, wherein said plant cell is obtained from a plant selected from the group consisting of *Arabidopsis*, soybean, and corn.

24. A method for producing a tocopherol compound of interest in a host cell, said method comprising obtaining a transformed host cell, said host cell having and expressing in its genome:

a construct having a DNA sequence encoding a prenyltransferase operably linked to a transcriptional initiation region functional in a host cell,

wherein said prenyltransferase is involved in the synthesis of tocopherols.

25. The method according to Claim 24, wherein said host cell is selected from the group consisting of a prokaryotic cell and a eukaryotic cell.

26. The method according to Claim 25, wherein said prokaryotic cell is *Synechocystis.*

27. The method according to Claim 24, wherein said eukaryotic cell is a plant cell.

28. The method according to Claim 27, wherein said plant cell is obtained from a plant selected from the group consisting of *Arabidopsis*, soybean, and corn.

29. A method for increasing the biosynthetic flux in cell from a host cell toward tocopherol production, said method comprising transforming said host cell with a construct comprising as operably linked components, a transcriptional initiation region functional in a

host cell, a DNA encoding a prenyltransferase involved in the synthesis of tocopherols, and a transcriptional termination region.

30. The method according to Claim 29, wherein said host cell is selected from the group consisting of a prokaryotic cell and a eukaryotic cell.

31. The method according to Claim 30, wherein said prokaryotic cell is *Synechocystis*.

32. The method according to Claim 30, wherein said eukaryotic cell is a plant cell.

33. The method according to Claim 32, wherein said plant cell is obtained from a plant selected from the group consisting of *Arabidopsis*, soybean, and corn.

```
                       *        20          *         40          *         60          *         80          *
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ATPT3  : MAFFGLSRVSRRLLKSSVSVTPSSSSALLQSQHKSLSNPVTTHYTNPFTKCYPSWNDNYQVWSKGRELHQEKKFGVGWNYRLICGMSSSS :  90
ATPT4  : -----MWRRS-VVYRFSSRISVSSSLPNPRLIPWSREL----CAVNSFSQPPVSTESTAKLGITGV-----RSD----ANRVFA--TATA :  69
ATPT8  : -------------MVLAEVPKLAS-----AAEYFFKR------GVQGKQFR----STILLLMATA------LN-----VRVPE--ALIG :  48
ATPT12 : -------MTS--IINTVSTIHSSRVTSVDRVGVLSLRN----SDSVEFTRRR-SGFSTLIYESPGR-----RFV-----VRAAE--TDTD :  64
                 s       6                                                                r

             100         *       120          *       140          *        160          *        180
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ATPT3  : SVLEGKPKKDDKEKSDGVVVKKASWIDLYLPEEVRGYAKLARLDKPIGTWLLAWPCMWSIAL-AADPGSLPSF--K------YMALFGCG : 171
ATPT4  : AATATAT----TG-EISSRVAALLAGLGHHYARCYWELSK-AKLSMLVVATS---GTGYVIGT-GNAAISFPGL----------C--YTCA : 137
ATPT8  : ESTDIVT----SELRVRQRGIREITEMIHVASLLHDDVL-DDADTRRGVGS---LNWVMGNKMSVLAGDFLLS-------------RACG : 117
ATPT12 : KVKSQTP---DKAPAGGSSINQLLGIKG-ASQETNKWKIRLQLTKPV-TWP---PLVWGVVCGAAASGNFHWTPED------VAKSILCM : 140
                                                         l                    f                 c

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ATPT8  : AL--AALKNTEVVALLATAVEHLVTGET---MEITSSTEQRYSMDYYMQKTYYKT---ASLISNSCK---------AVAVLLGQTAEVAV : 190
ATPT12 : MMSGPCLTGYTQTINDWYDRDIDAINEP--YRPIPSGAISEPEVITQVWVLLLGG-LGIAGILDVWAGHTTPTVFYLALGGSLLSYIYSA : 227
              6        n        d           p6 Sg

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ATPT8  : LAFEYGRNLGLAFQLI----DDILDFTGTS----ASLGKGSLSDIRHGVIPAPILFAMEEFPQLREVVDQVEK--------DP-----RN : 259
ATPT12 : PPLKLKQNGWVGNFA-LG--ASYISLPWWAGQ--ALFGTLTPDVVVL---LLYSIAG-LGIAIVNDFKSVEG--------D------RA : 294
                                           a G                                 e         D

                 *        380         *       400          *       420          *       440          *
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ATPT3  : VGVKS----TALRFGD----NTKLWLTGFGTASIGFLALSGFSADLGWQYYAS----LAAASGQLGWQIGTADLSSGADCSRKFVSNKWF : 392
ATPT4  : VALRNCFYMIPLGFIAYDWGLISSWFCLESTLLTNAIAATAFSFYRDRTMHKARKMFHASLLFLPVFMSGLLLHRVSNDNQQQLVEEAGL : 390
ATPT8  : VDIAL----EYLGKSK--------GLQ---RARELAMEHANLAAAAIGSLPET----DNEDVKRSRRALIDLTHRVITRNK--------- : 321
ATPT12 : LGLQS----LPVAFGT----ETAKWIC-VGAIDITQLSVAGYLLASGKPYYALA--LVALLIPQIVFQFKYFLKDPVKYDVKYQASAQPF : 373
          6        6             w

            460         *       480          *
ATPT2  : -MFIWKLFYAE----YLLLPFLK------------------- : 393
ATPT3  : GAIIFSGVVLG----RSFQ----------------------- : 407
ATPT4  : TNSVSGEVKTQRRKKRVAQPPVAYASAAPFPFLPAPSFYSP : 431
ATPT8  : ------------------------------------------ :   -
ATPT12 : --LVLGIFVTA----LASQH---------------------- : 387
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Figure 1

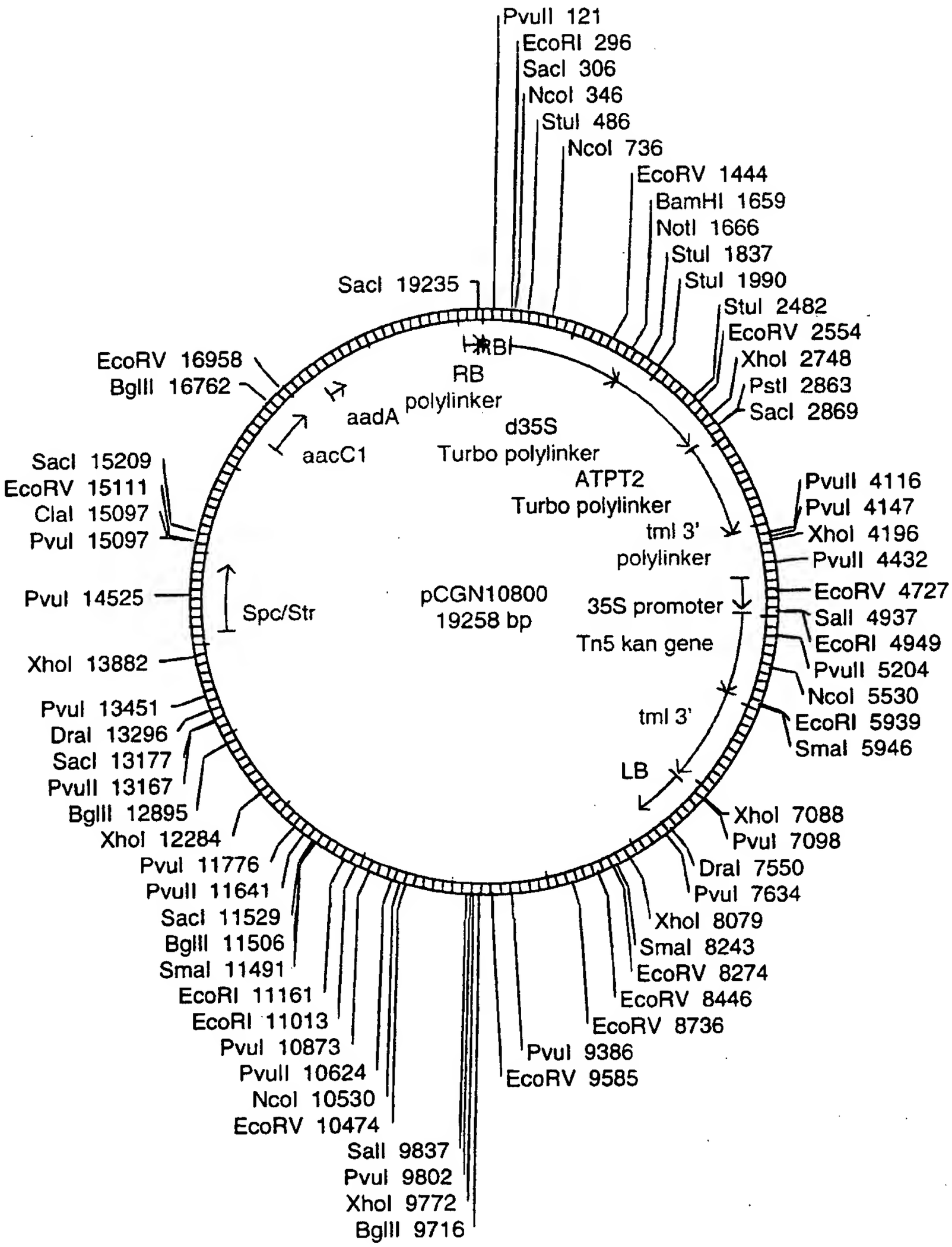
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Figure 2

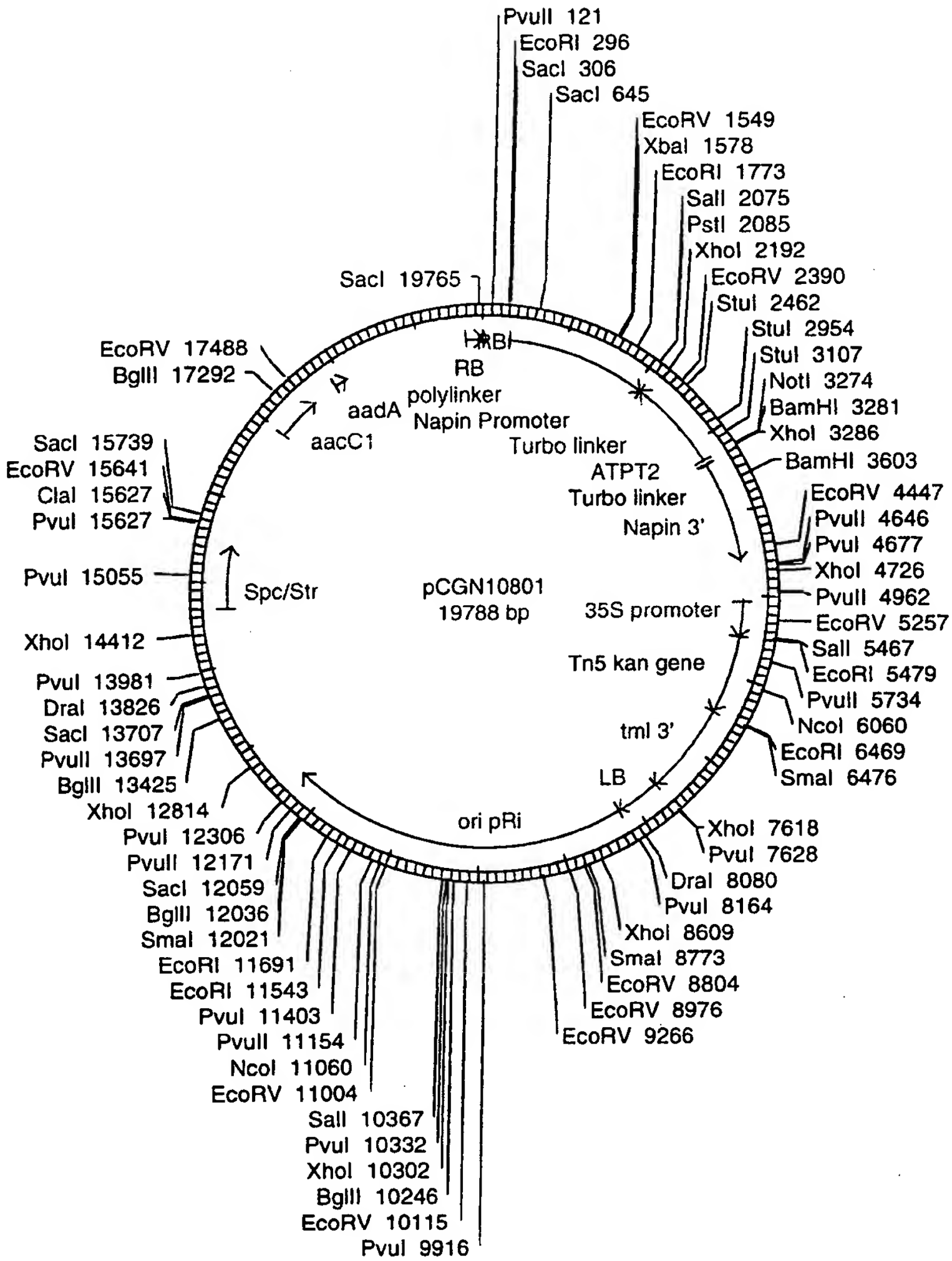
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Figure 3

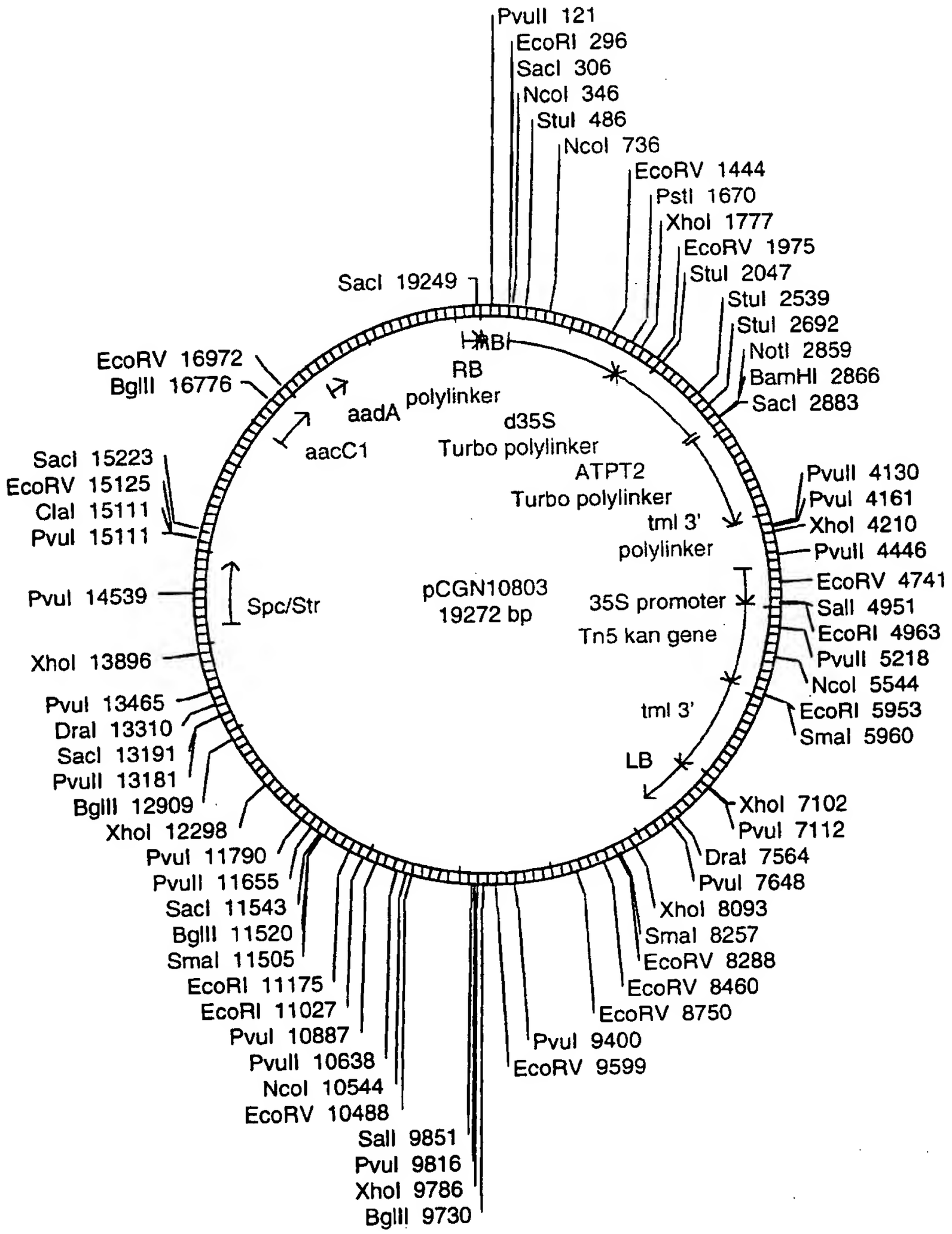
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Figure 4

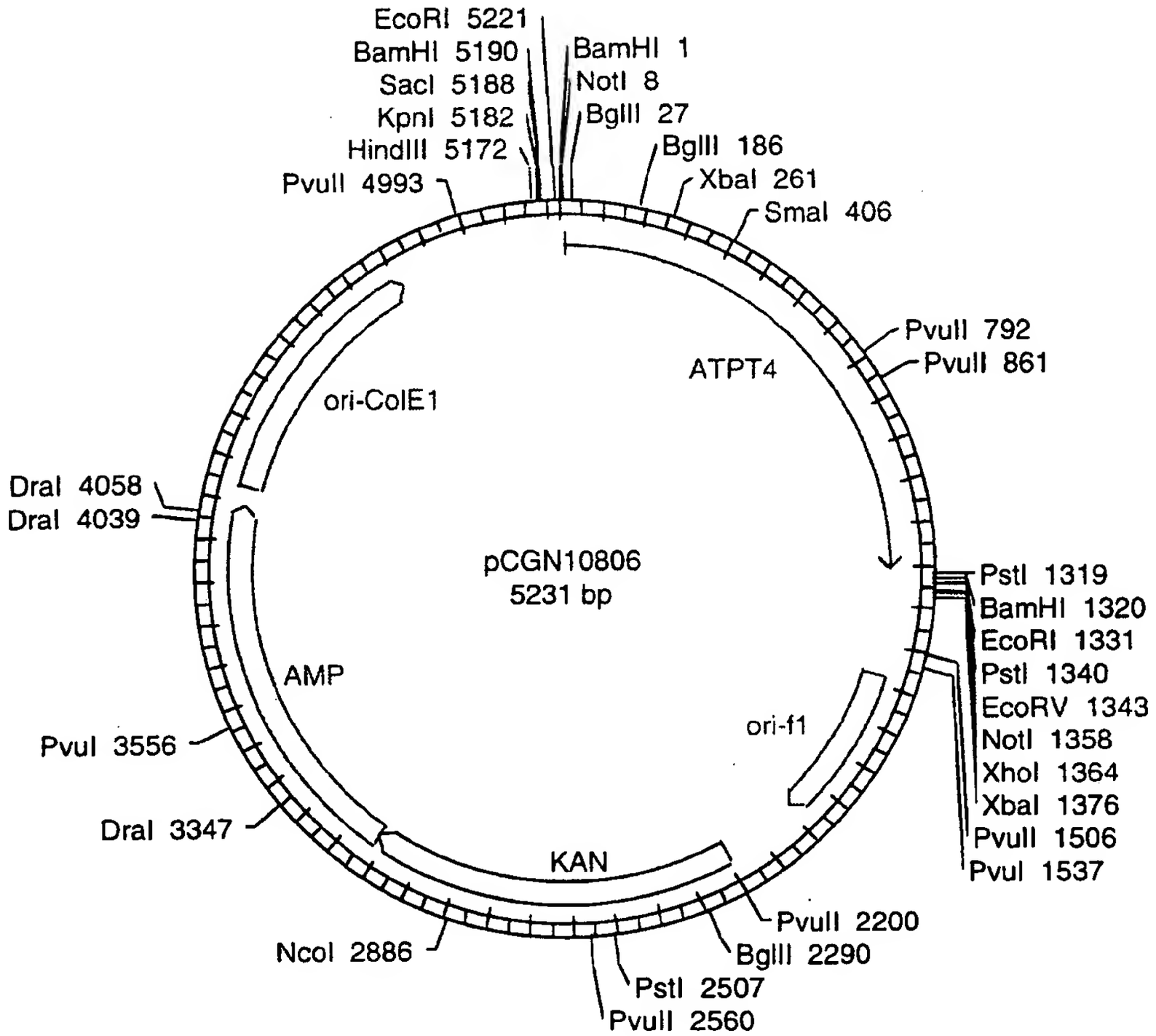
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Figure 5

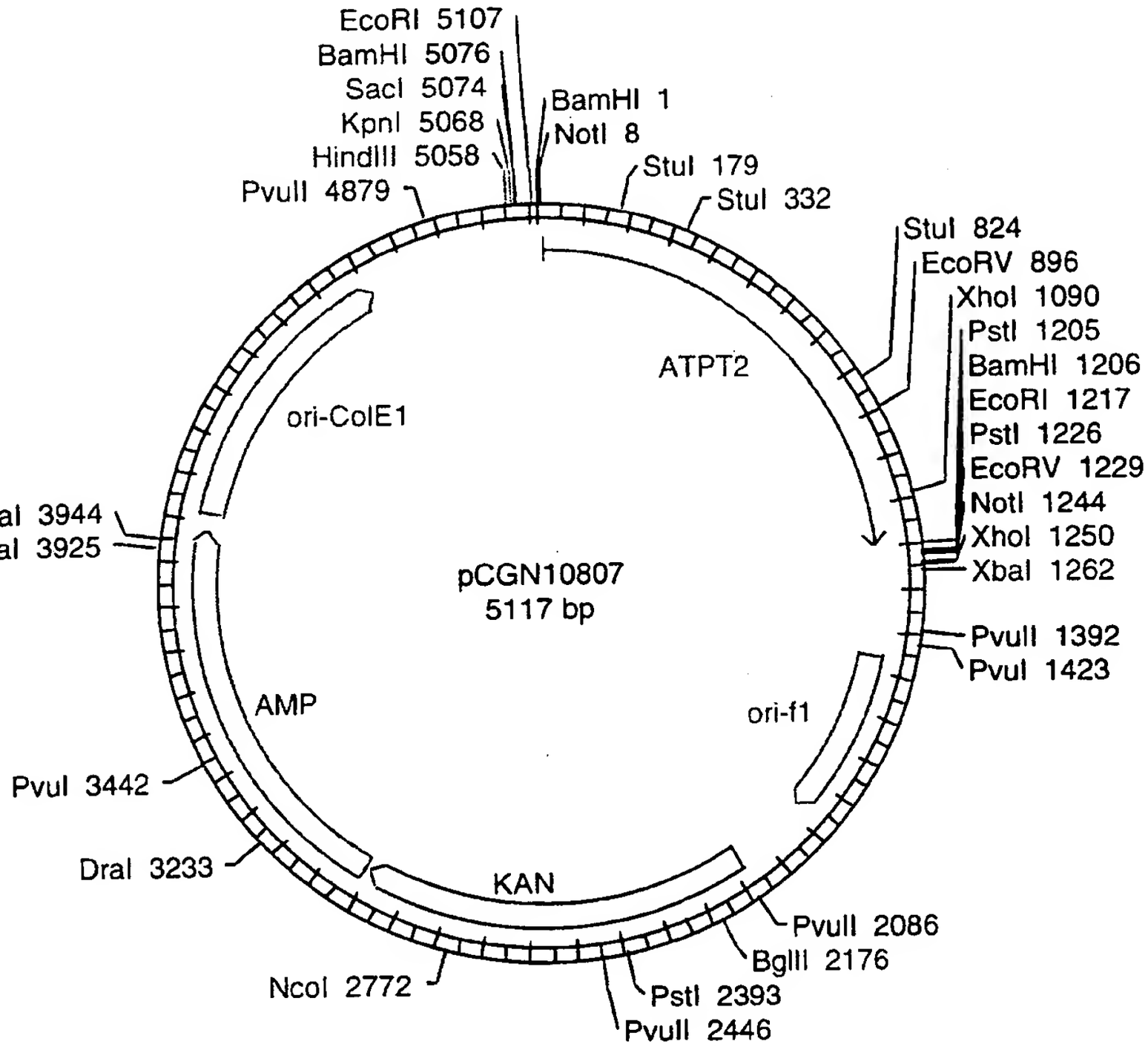
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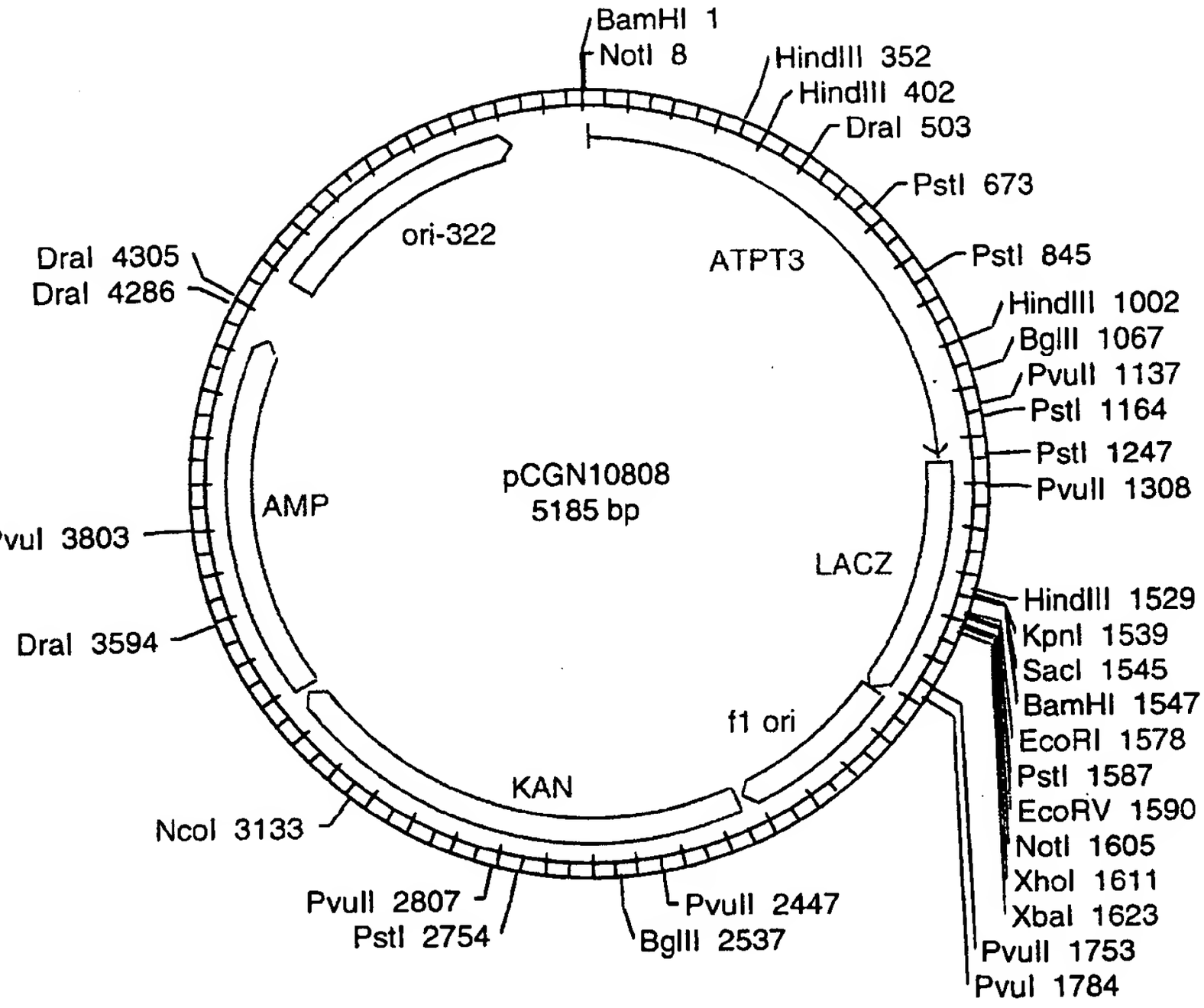
Figure 6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KAN

Figure 7

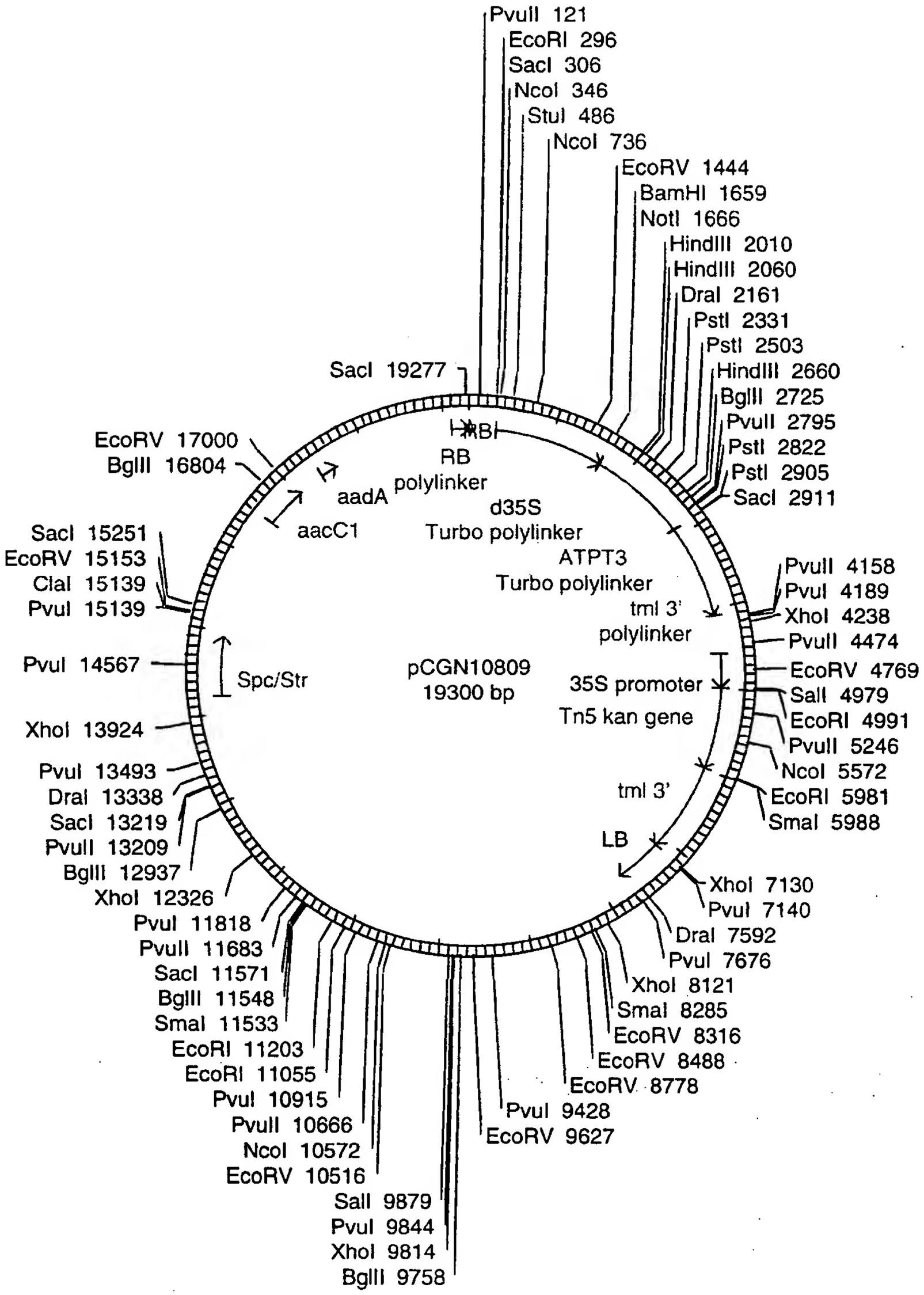

PvuII 121
EcoRI 296
SacI 306
NcoI 346
StuI 486
NcoI 736
EcoRV 1444
BamHI 1659
NotI 1666
HindIII 2010
HindIII 2060
DraI 2161
PstI 2331
PstI 2503
HindIII 2660
BglII 2725
PvuII 2795
PstI 2822
PstI 2905
SacI 2911
SacI 19277
RB
RB
polylinker
aadA
aacC1
d35S
Turbo polylinker
ATPT3
Turbo polylinker
tml 3'
polylinker
35S promoter
Tn5 kan gene
tml 3'
LB
pCGN10809
19300 bp
Spc/Str
EcoRV 17000
BglII 16804
SacI 15251
EcoRV 15153
ClaI 15139
PvuI 15139
PvuI 14567
XhoI 13924
PvuI 13493
DraI 13338
SacI 13219
PvuII 13209
BglII 12937
XhoI 12326
PvuI 11818
PvuII 11683
SacI 11571
BglII 11548
SmaI 11533
EcoRI 11203
EcoRI 11055
PvuI 10915
PvuII 10666
NcoI 10572
EcoRV 10516
SalI 9879
PvuI 9844
XhoI 9814
BglII 9758
PvuI 9428
EcoRV 9627
EcoRV 8778
EcoRV 8488
EcoRV 8316
SmaI 8285
XhoI 8121
DraI 7592
PvuI 7676
XhoI 7130
PvuI 7140
PvuII 4158
PvuI 4189
XhoI 4238
PvuII 4474
EcoRV 4769
SalI 4979
EcoRI 4991
PvuII 5246
NcoI 5572
EcoRI 5981
SmaI 5988

Figure 8

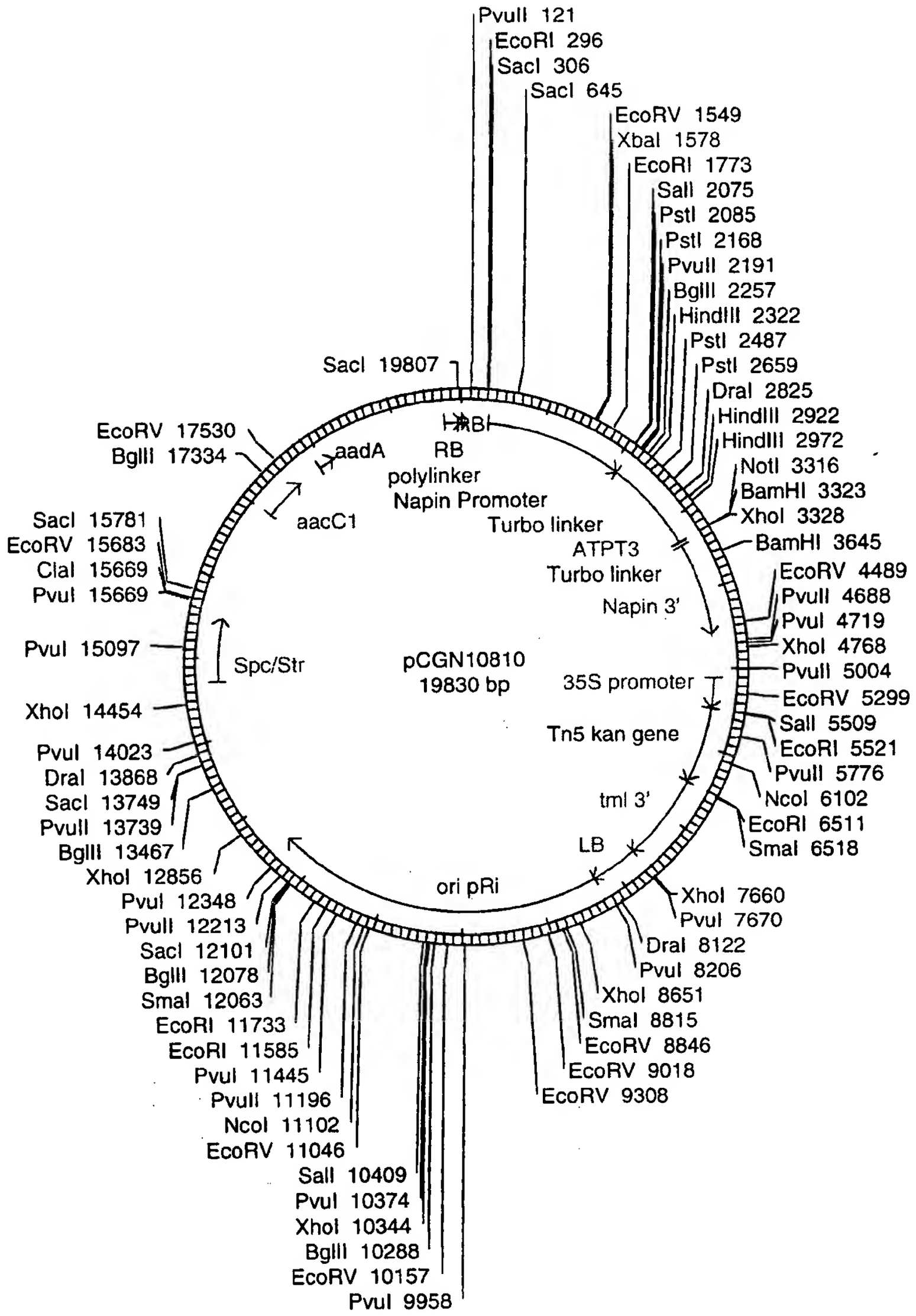

PvuII 121
EcoRI 296
SacI 306
SacI 645
EcoRV 1549
XbaI 1578
EcoRI 1773
SalI 2075
PstI 2085
PstI 2168
PvuII 2191
BglII 2257
HindIII 2322
PstI 2487
PstI 2659
DraI 2825
HindIII 2922
HindIII 2972
NotI 3316
BamHI 3323
XhoI 3328
BamHI 3645
EcoRV 4489
PvuII 4688
PvuI 4719
XhoI 4768
PvuII 5004
EcoRV 5299
SalI 5509
EcoRI 5521
PvuII 5776
NcoI 6102
EcoRI 6511
SmaI 6518
XhoI 7660
PvuI 7670
DraI 8122
PvuI 8206
XhoI 8651
SmaI 8815
EcoRV 8846
EcoRV 9018
EcoRV 9308
SacI 19807
EcoRV 17530
BglII 17334
SacI 15781
EcoRV 15683
ClaI 15669
PvuI 15669
PvuI 15097
XhoI 14454
PvuI 14023
DraI 13868
SacI 13749
PvuII 13739
BglII 13467
XhoI 12856
PvuI 12348
PvuII 12213
SacI 12101
BglII 12078
SmaI 12063
EcoRI 11733
EcoRI 11585
PvuI 11445
PvuII 11196
NcoI 11102
EcoRV 11046
SalI 10409
PvuI 10374
XhoI 10344
BglII 10288
EcoRV 10157
PvuI 9958
aadA
aacC1
Spc/Str
RB
polylinker
Napin Promoter
Turbo linker
ATPT3
Turbo linker
Napin 3'
35S promoter
Tn5 kan gene
tml 3'
LB
ori pRi
pCGN10810
19830 bp

Figure 9

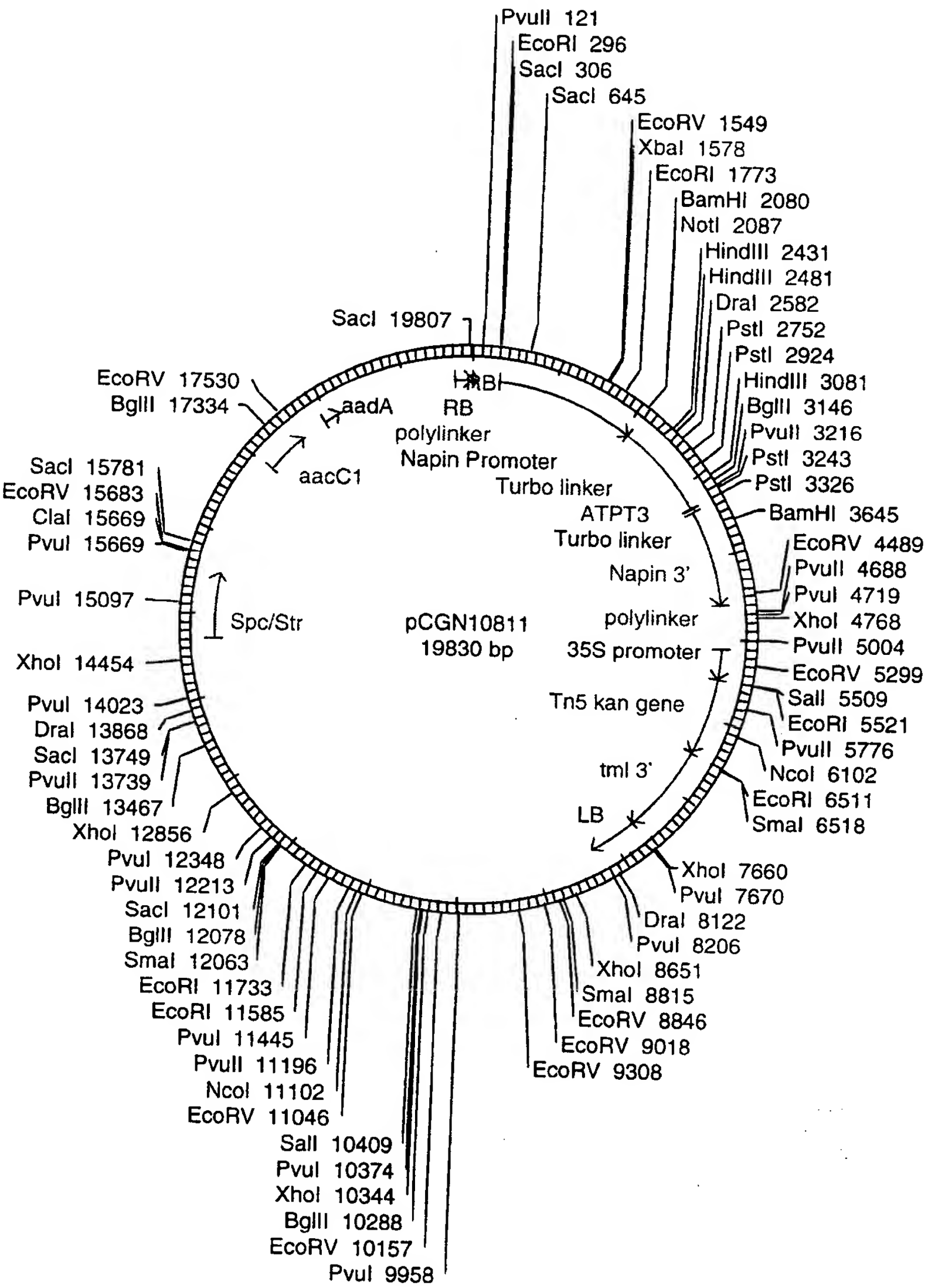

PvuII 121
EcoRI 296
SacI 306
SacI 645
EcoRV 1549
XbaI 1578
EcoRI 1773
BamHI 2080
NotI 2087
HindIII 2431
HindIII 2481
DraI 2582
PstI 2752
PstI 2924
HindIII 3081
BglII 3146
PvuII 3216
PstI 3243
PstI 3326
BamHI 3645
EcoRV 4489
PvuII 4688
PvuI 4719
XhoI 4768
PvuII 5004
EcoRV 5299
SalI 5509
EcoRI 5521
PvuII 5776
NcoI 6102
EcoRI 6511
SmaI 6518
XhoI 7660
PvuI 7670
DraI 8122
PvuI 8206
XhoI 8651
SmaI 8815
EcoRV 8846
EcoRV 9018
EcoRV 9308
SacI 19807
EcoRV 17530
BglII 17334
SacI 15781
EcoRV 15683
ClaI 15669
PvuI 15669
PvuI 15097
XhoI 14454
PvuI 14023
DraI 13868
SacI 13749
PvuII 13739
BglII 13467
XhoI 12856
PvuI 12348
PvuII 12213
SacI 12101
BglII 12078
SmaI 12063
EcoRI 11733
EcoRI 11585
PvuI 11445
PvuII 11196
NcoI 11102
EcoRV 11046
SalI 10409
PvuI 10374
XhoI 10344
BglII 10288
EcoRV 10157
PvuI 9958
RB
aadA
polylinker
Napin Promoter
Turbo linker
ATPT3
Turbo linker
Napin 3'
polylinker
35S promoter
Tn5 kan gene
tml 3'
LB
aacC1
Spc/Str
pCGN10811
19830 bp

Figure 10

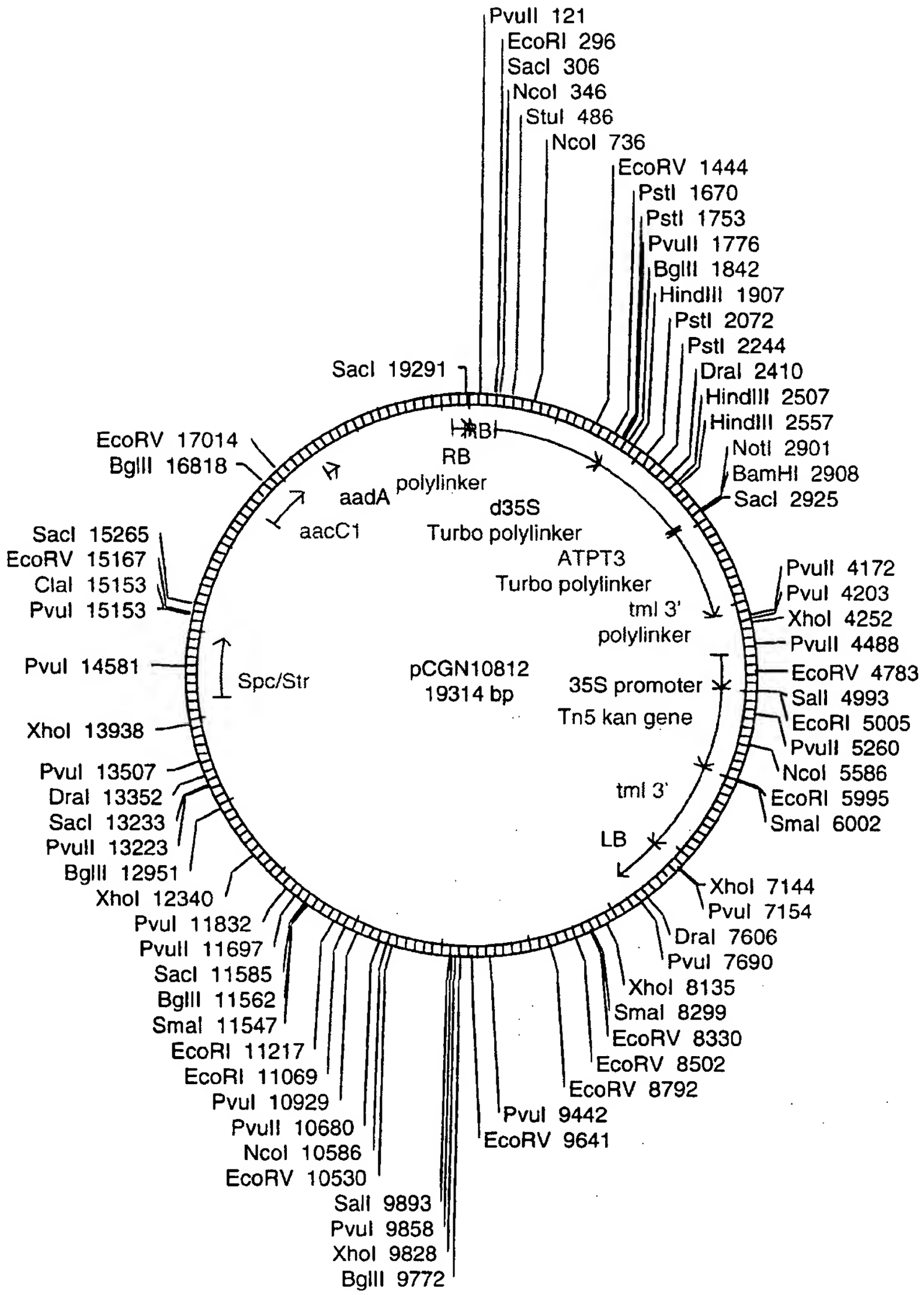

PvuII 121
EcoRI 296
SacI 306
NcoI 346
StuI 486
NcoI 736
EcoRV 1444
PstI 1670
PstI 1753
PvuII 1776
BglII 1842
HindIII 1907
PstI 2072
PstI 2244
DraI 2410
HindIII 2507
HindIII 2557
NotI 2901
BamHI 2908
SacI 2925
PvuII 4172
PvuI 4203
XhoI 4252
PvuII 4488
EcoRV 4783
SalI 4993
EcoRI 5005
PvuII 5260
NcoI 5586
EcoRI 5995
SmaI 6002
XhoI 7144
PvuI 7154
DraI 7606
PvuI 7690
XhoI 8135
SmaI 8299
EcoRV 8330
EcoRV 8502
EcoRV 8792
PvuI 9442
EcoRV 9641
SalI 9893
PvuI 9858
XhoI 9828
BglII 9772
XhoI 12340
PvuI 11832
PvuII 11697
SacI 11585
BglII 11562
SmaI 11547
EcoRI 11217
EcoRI 11069
PvuI 10929
PvuII 10680
NcoI 10586
EcoRV 10530
DraI 13352
SacI 13233
PvuII 13223
BglII 12951
PvuI 13507
XhoI 13938
PvuI 14581
SacI 15265
EcoRV 15167
ClaI 15153
PvuI 15153
EcoRV 17014
BglII 16818
SacI 19291
RB
polylinker
aadA
aacC1
Spc/Str
d35S
Turbo polylinker
ATPT3
Turbo polylinker
tml 3'
polylinker
35S promoter
Tn5 kan gene
tml 3'
LB
pCGN10812
19314 bp

Figure 11

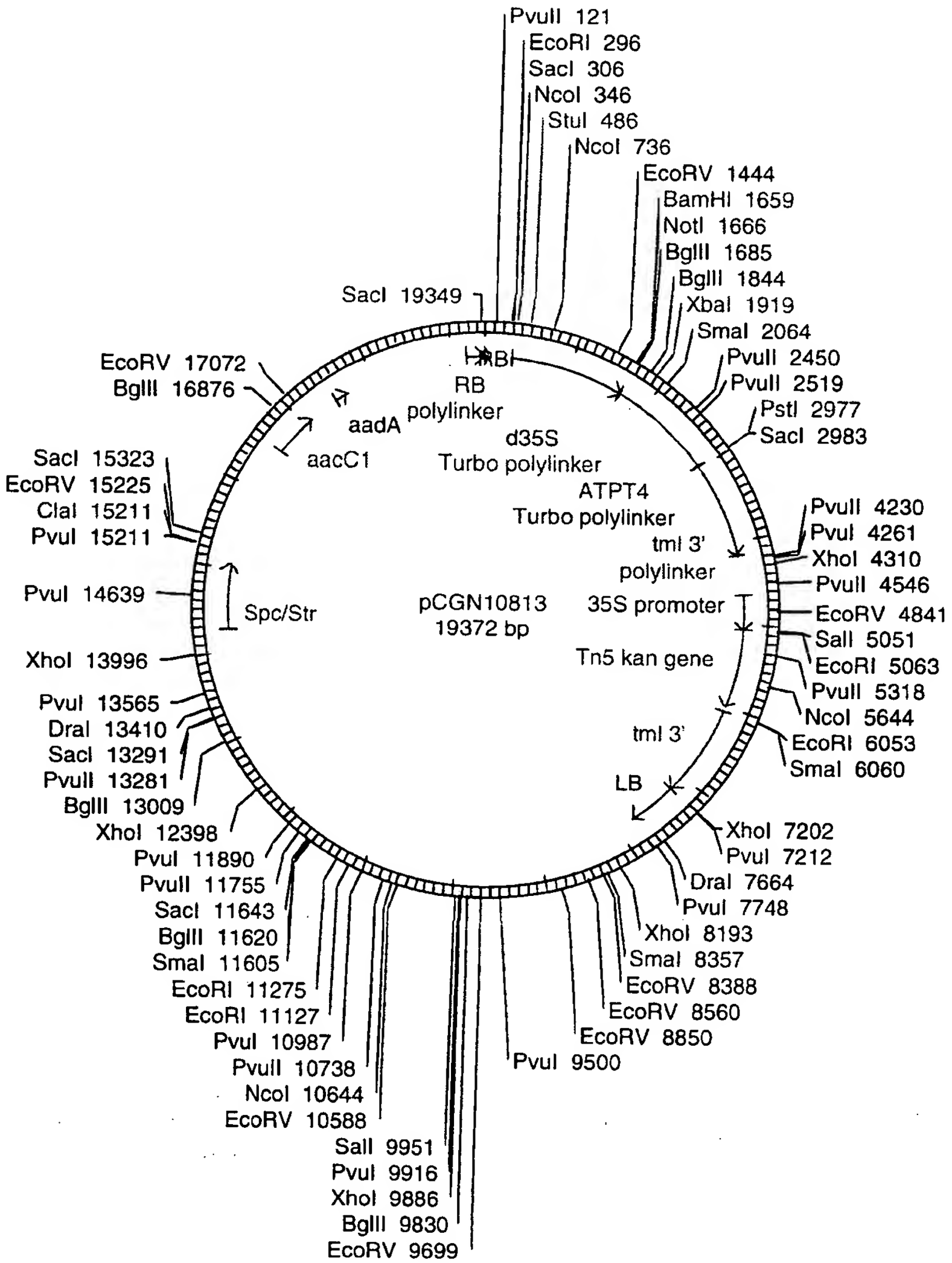

PvuII 121
EcoRI 296
SacI 306
NcoI 346
StuI 486
NcoI 736
EcoRV 1444
BamHI 1659
NotI 1666
BglII 1685
BglII 1844
XbaI 1919
SmaI 2064
PvuII 2450
PvuII 2519
PstI 2977
SacI 2983
PvuII 4230
PvuI 4261
XhoI 4310
PvuII 4546
EcoRV 4841
SalI 5051
EcoRI 5063
PvuII 5318
NcoI 5644
EcoRI 6053
SmaI 6060
XhoI 7202
PvuI 7212
DraI 7664
PvuI 7748
XhoI 8193
SmaI 8357
EcoRV 8388
EcoRV 8560
EcoRV 8850
PvuI 9500
SalI 9951
PvuI 9916
XhoI 9886
BglII 9830
EcoRV 9699
SacI 11643
BglII 11620
SmaI 11605
EcoRI 11275
EcoRI 11127
PvuI 10987
PvuII 10738
NcoI 10644
EcoRV 10588
PvuI 11890
PvuII 11755
DraI 13410
SacI 13291
PvuII 13281
BglII 13009
XhoI 12398
PvuI 13565
XhoI 13996
PvuI 14639
SacI 15323
EcoRV 15225
ClaI 15211
PvuI 15211
SacI 19349
EcoRV 17072
BglII 16876
RB
RB
polylinker
aadA
aacC1
d35S
Turbo polylinker
ATPT4
Turbo polylinker
tml 3'
polylinker
35S promoter
Tn5 kan gene
tml 3'
LB
pCGN10813
19372 bp
Spc/Str

Figure 12

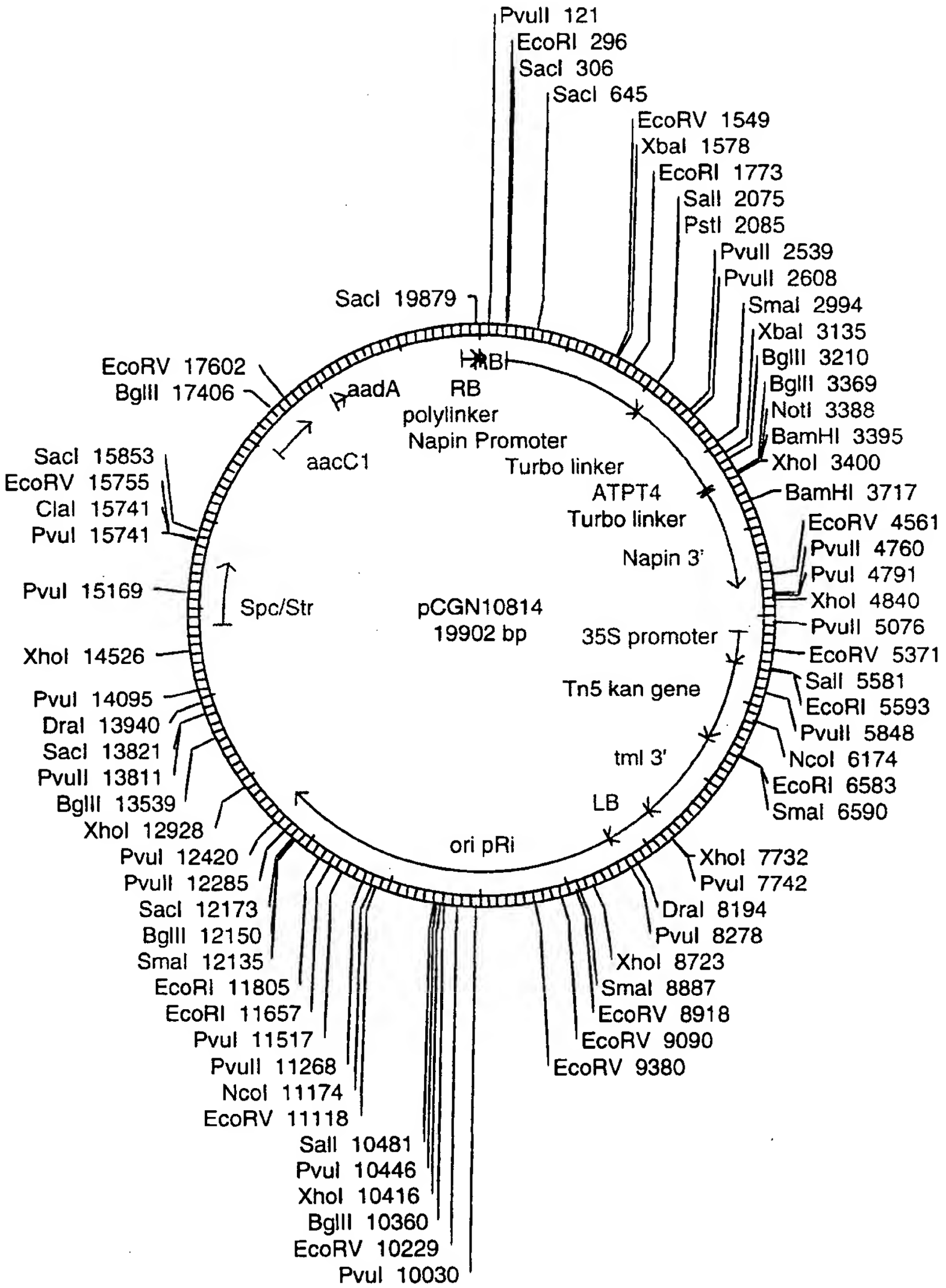

pCGN10814
19902 bp
PvuII 121
EcoRI 296
SacI 306
SacI 645
EcoRV 1549
XbaI 1578
EcoRI 1773
SalI 2075
PstI 2085
PvuII 2539
PvuII 2608
SmaI 2994
XbaI 3135
BglII 3210
BglII 3369
NotI 3388
BamHI 3395
XhoI 3400
BamHI 3717
EcoRV 4561
PvuII 4760
PvuI 4791
XhoI 4840
PvuII 5076
EcoRV 5371
SalI 5581
EcoRI 5593
PvuII 5848
NcoI 6174
EcoRI 6583
SmaI 6590
XhoI 7732
PvuI 7742
DraI 8194
PvuI 8278
XhoI 8723
SmaI 8887
EcoRV 8918
EcoRV 9090
EcoRV 9380
SalI 10481
PvuI 10446
XhoI 10416
BglII 10360
EcoRV 10229
PvuI 10030
NcoI 11174
EcoRV 11118
PvuII 11268
PvuI 11517
EcoRI 11657
EcoRI 11805
SmaI 12135
BglII 12150
SacI 12173
PvuII 12285
PvuI 12420
XhoI 12928
BglII 13539
PvuII 13811
SacI 13821
DraI 13940
PvuI 14095
XhoI 14526
PvuI 15169
ClaI 15741
PvuI 15741
EcoRV 15755
SacI 15853
EcoRV 17602
BglII 17406
SacI 19879
aadA
RB
polylinker
Napin Promoter
aacC1
Turbo linker
ATPT4
Turbo linker
Napin 3'
35S promoter
Tn5 kan gene
tml 3'
LB
ori pRi
Spc/Str

Figure 13

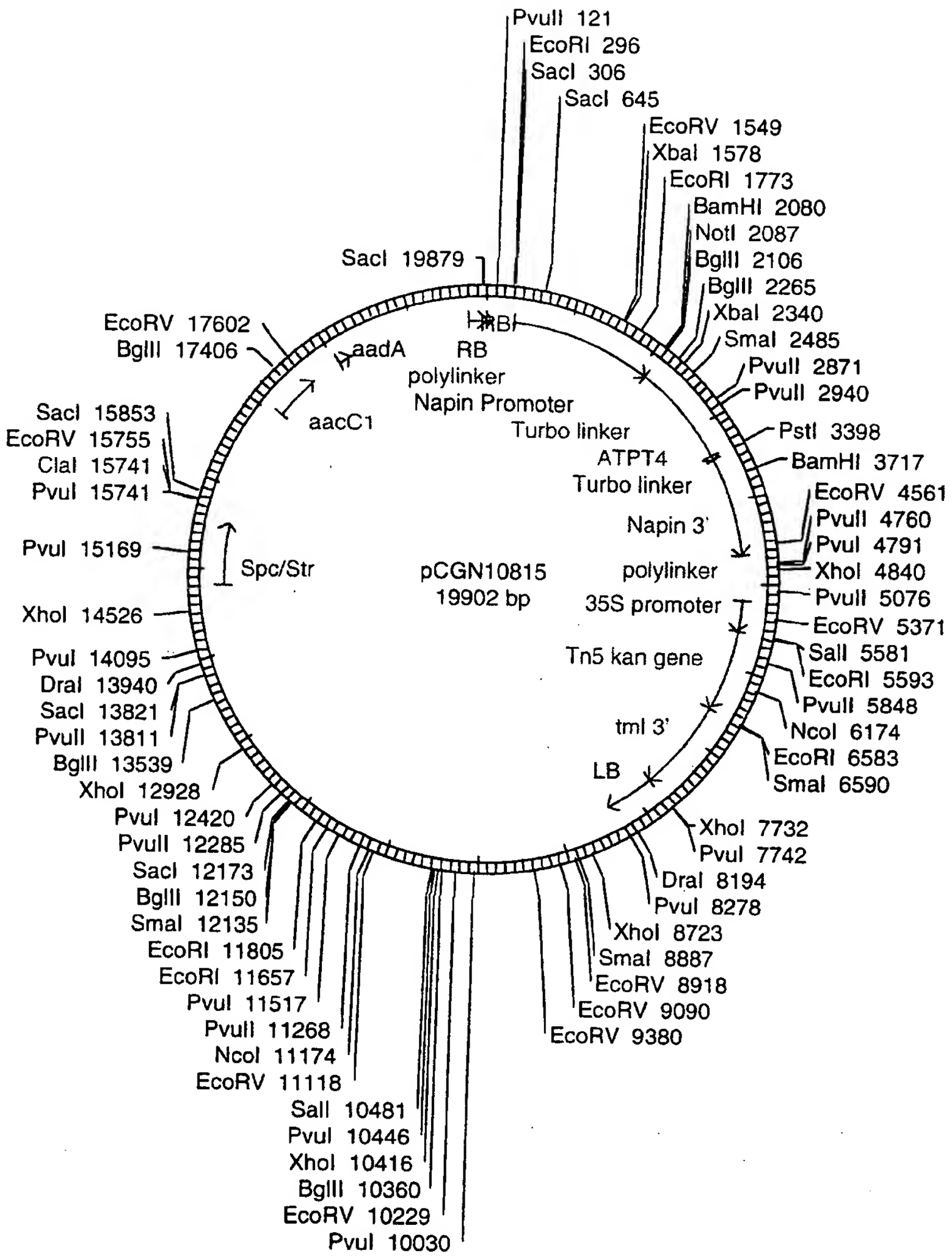

PvuII 121
EcoRI 296
SacI 306
SacI 645
EcoRV 1549
XbaI 1578
EcoRI 1773
BamHI 2080
NotI 2087
BglII 2106
BglII 2265
XbaI 2340
SmaI 2485
PvuII 2871
PvuII 2940
PstI 3398
BamHI 3717
EcoRV 4561
PvuII 4760
PvuI 4791
XhoI 4840
PvuII 5076
EcoRV 5371
SalI 5581
EcoRI 5593
PvuII 5848
NcoI 6174
EcoRI 6583
SmaI 6590
XhoI 7732
PvuI 7742
DraI 8194
PvuI 8278
XhoI 8723
SmaI 8887
EcoRV 8918
EcoRV 9090
EcoRV 9380
SacI 19879
EcoRV 17602
BglII 17406
SacI 15853
EcoRV 15755
ClaI 15741
PvuI 15741
PvuI 15169
XhoI 14526
PvuI 14095
DraI 13940
SacI 13821
PvuII 13811
BglII 13539
XhoI 12928
PvuI 12420
PvuII 12285
SacI 12173
BglII 12150
SmaI 12135
EcoRI 11805
EcoRI 11657
PvuI 11517
PvuII 11268
NcoI 11174
EcoRV 11118
SalI 10481
PvuI 10446
XhoI 10416
BglII 10360
EcoRV 10229
PvuI 10030
RB
aadA
aacC1
polylinker
Napin Promoter
Turbo linker
ATPT4
Turbo linker
Napin 3'
polylinker
35S promoter
Tn5 kan gene
tml 3'
LB
Spc/Str
pCGN10815
19902 bp

Figure 14

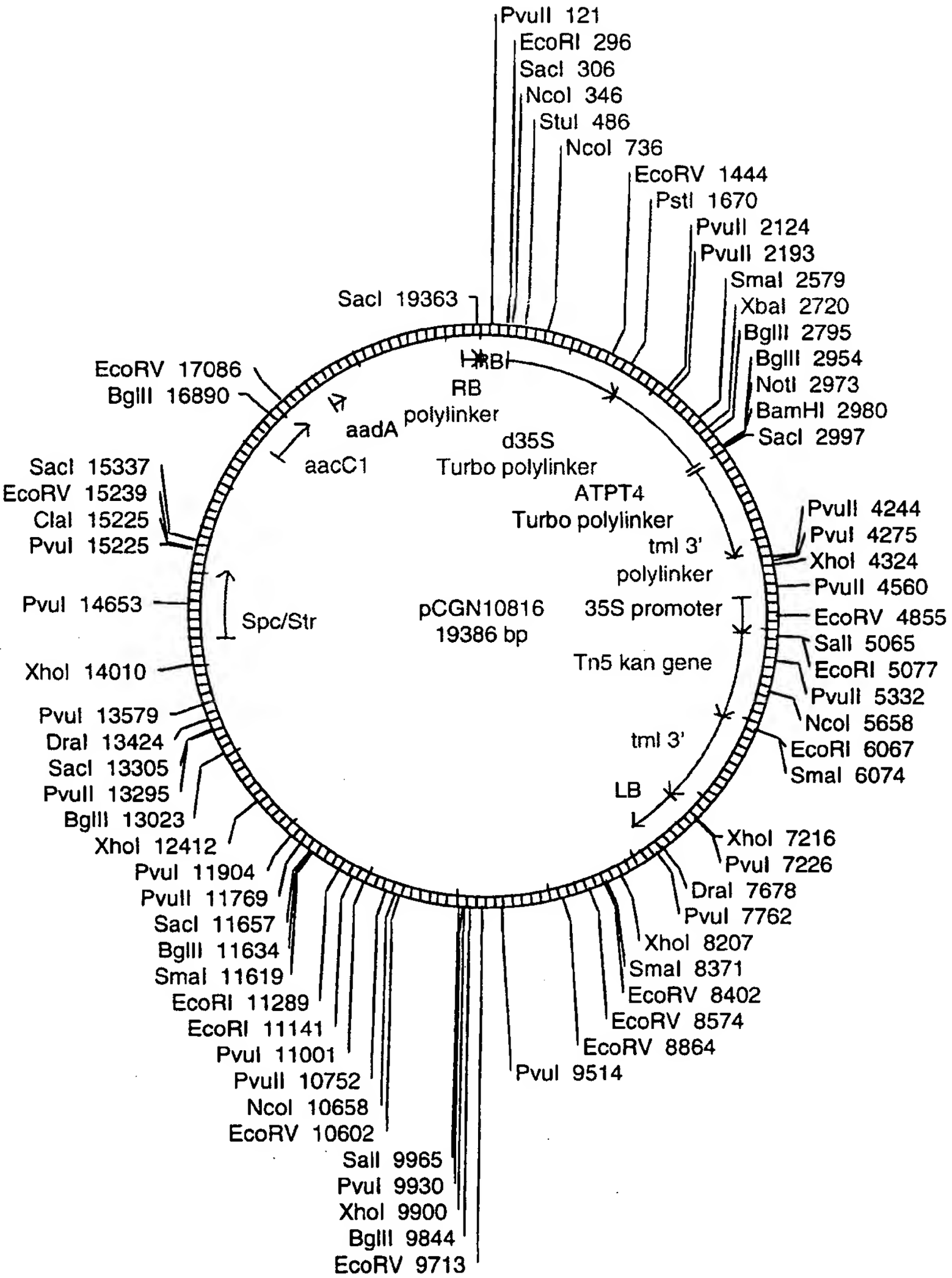

PvuII 121
EcoRI 296
SacI 306
NcoI 346
StuI 486
NcoI 736
EcoRV 1444
PstI 1670
PvuII 2124
PvuII 2193
SmaI 2579
XbaI 2720
BglII 2795
BglII 2954
NotI 2973
BamHI 2980
SacI 2997
SacI 19363
RB
RB polylinker
d35S Turbo polylinker
ATPT4 Turbo polylinker
tml 3' polylinker
35S promoter
Tn5 kan gene
tml 3'
LB
pCGN10816
19386 bp
EcoRV 17086
BglII 16890
aadA
aacC1
Spc/Str
SacI 15337
EcoRV 15239
ClaI 15225
PvuI 15225
PvuI 14653
XhoI 14010
PvuI 13579
DraI 13424
SacI 13305
PvuII 13295
BglII 13023
XhoI 12412
PvuI 11904
PvuII 11769
SacI 11657
BglII 11634
SmaI 11619
EcoRI 11289
EcoRI 11141
PvuI 11001
PvuII 10752
NcoI 10658
EcoRV 10602
SalI 9965
PvuI 9930
XhoI 9900
BglII 9844
EcoRV 9713
PvuI 9514
EcoRV 8864
EcoRV 8574
EcoRV 8402
SmaI 8371
XhoI 8207
PvuI 7762
DraI 7678
PvuI 7226
XhoI 7216
EcoRI 6067
SmaI 6074
NcoI 5658
PvuII 5332
EcoRI 5077
SalI 5065
EcoRV 4855
PvuII 4560
XhoI 4324
PvuI 4275
PvuII 4244

Figure 15

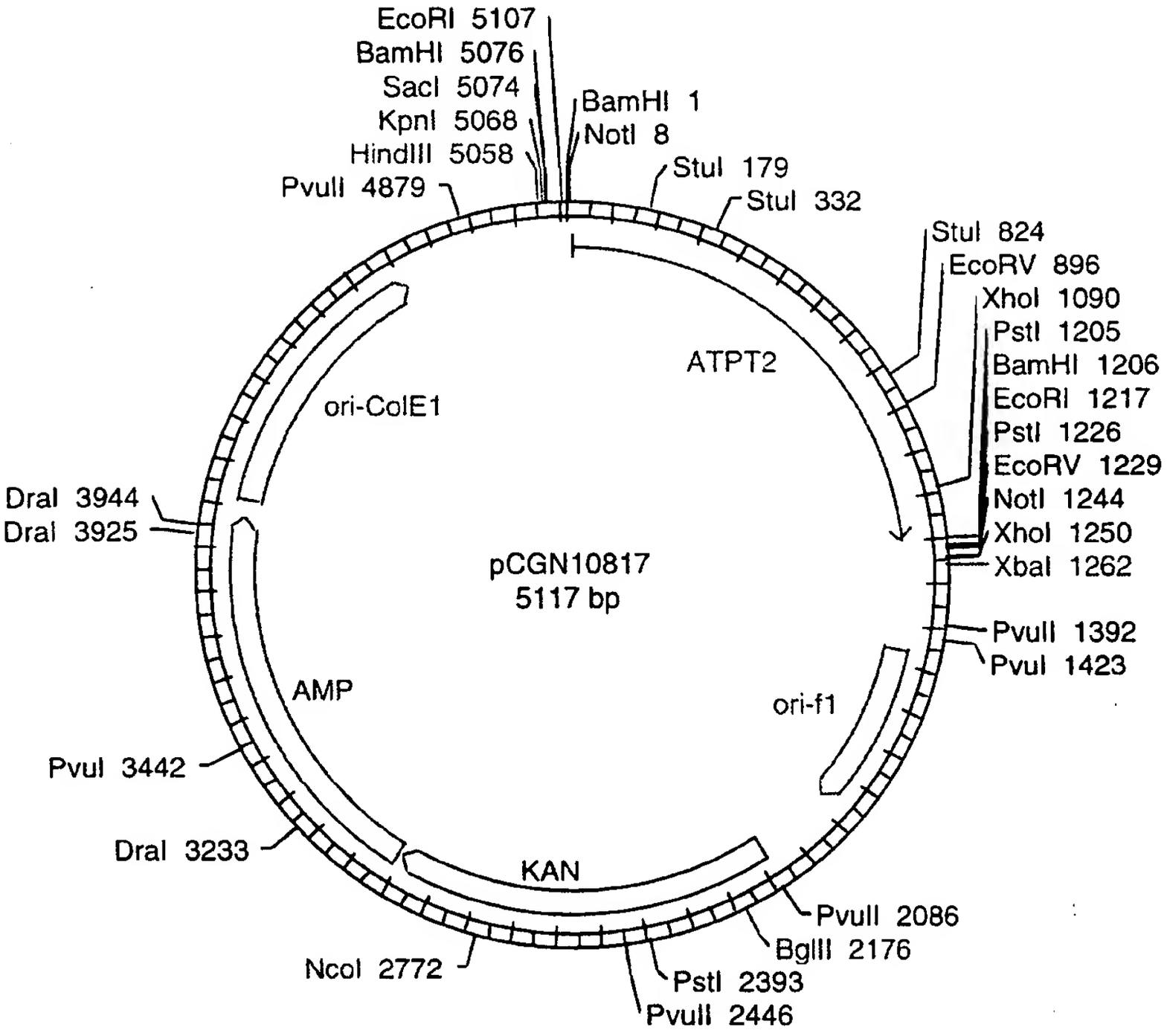

EcoRI 5107
BamHI 5076
SacI 5074
KpnI 5068
HindIII 5058
PvuII 4879
BamHI 1
NotI 8
StuI 179
StuI 332
StuI 824
EcoRV 896
XhoI 1090
PstI 1205
BamHI 1206
EcoRI 1217
PstI 1226
EcoRV 1229
NotI 1244
XhoI 1250
XbaI 1262
PvuII 1392
PvuI 1423
ATPT2
ori-ColE1
DraI 3944
DraI 3925
pCGN10817
5117 bp
AMP
ori-f1
PvuI 3442
DraI 3233
KAN
PvuII 2086
BglII 2176
NcoI 2772
PstI 2393
PvuII 2446

Figure 16

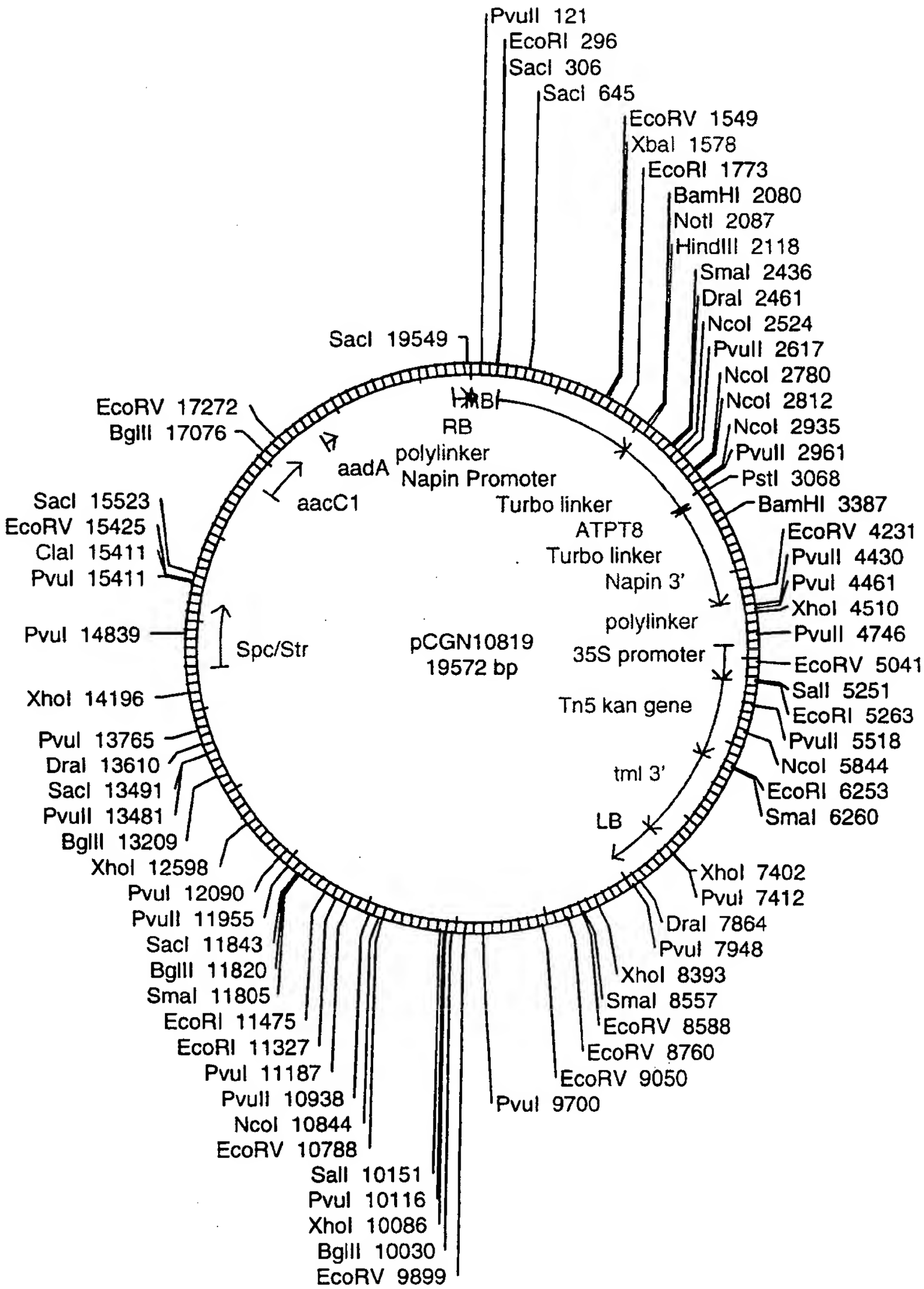

PvuII 121
EcoRI 296
SacI 306
SacI 645
EcoRV 1549
XbaI 1578
EcoRI 1773
BamHI 2080
NotI 2087
HindIII 2118
SmaI 2436
DraI 2461
NcoI 2524
PvuII 2617
NcoI 2780
NcoI 2812
NcoI 2935
PvuII 2961
PstI 3068
BamHI 3387
EcoRV 4231
PvuII 4430
PvuI 4461
XhoI 4510
PvuII 4746
EcoRV 5041
SalI 5251
EcoRI 5263
PvuII 5518
NcoI 5844
EcoRI 6253
SmaI 6260
XhoI 7402
PvuI 7412
DraI 7864
PvuI 7948
XhoI 8393
SmaI 8557
EcoRV 8588
EcoRV 8760
EcoRV 9050
PvuI 9700
SalI 10151
PvuI 10116
XhoI 10086
BglII 10030
EcoRV 9899
PvuII 10938
NcoI 10844
EcoRV 10788
PvuI 11187
EcoRI 11475
EcoRI 11327
SmaI 11805
BglII 11820
SacI 11843
PvuII 11955
PvuI 12090
XhoI 12598
BglII 13209
PvuII 13481
SacI 13491
DraI 13610
PvuI 13765
XhoI 14196
PvuI 14839
PvuI 15411
EcoRV 15425
ClaI 15411
SacI 15523
BglII 17076
EcoRV 17272
SacI 19549
RB
RB
polylinker
Napin Promoter
Turbo linker
ATPT8
Turbo linker
Napin 3'
polylinker
35S promoter
Tn5 kan gene
tml 3'
LB
aadA
aacC1
Spc/Str
pCGN10819
19572 bp

Figure 17

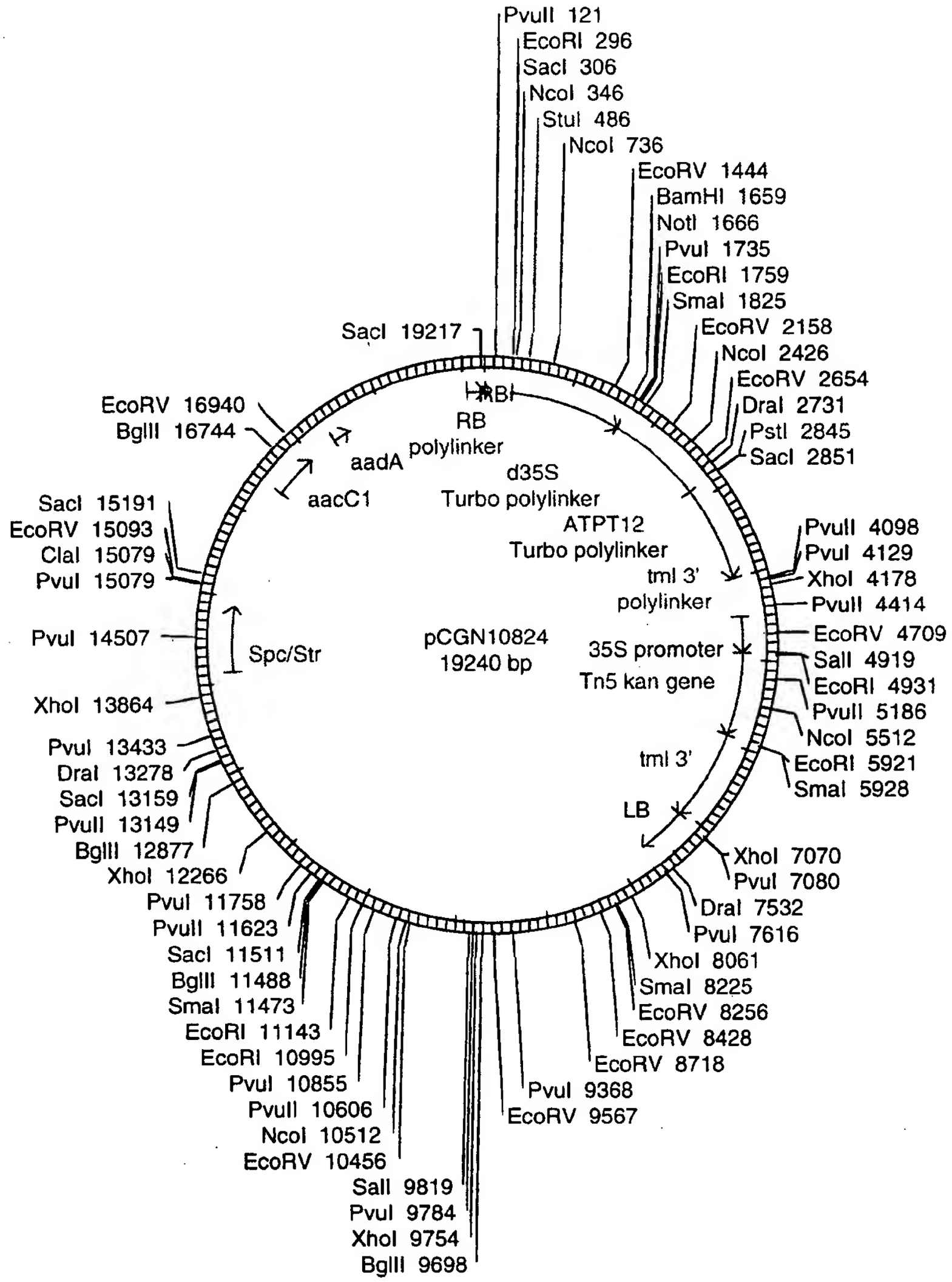

PvuII 121
EcoRI 296
SacI 306
NcoI 346
StuI 486
NcoI 736
EcoRV 1444
BamHI 1659
NotI 1666
PvuI 1735
EcoRI 1759
SmaI 1825
EcoRV 2158
NcoI 2426
EcoRV 2654
DraI 2731
PstI 2845
SacI 2851
SacI 19217
RB
RB polylinker
d35S Turbo polylinker
ATPT12 Turbo polylinker
tml 3' polylinker
35S promoter
Tn5 kan gene
tml 3'
LB
pCGN10824
19240 bp
EcoRV 16940
BglII 16744
aadA
aacC1
SacI 15191
EcoRV 15093
ClaI 15079
PvuI 15079
PvuI 14507
Spc/Str
XhoI 13864
PvuI 13433
DraI 13278
SacI 13159
PvuII 13149
BglII 12877
XhoI 12266
PvuI 11758
PvuII 11623
SacI 11511
BglII 11488
SmaI 11473
EcoRI 11143
EcoRI 10995
PvuI 10855
PvuII 10606
NcoI 10512
EcoRV 10456
SalI 9819
PvuI 9784
XhoI 9754
BglII 9698
PvuI 9368
EcoRV 9567
EcoRV 8718
EcoRV 8428
EcoRV 8256
SmaI 8225
XhoI 8061
PvuI 7616
DraI 7532
PvuI 7080
XhoI 7070
PvuII 4098
PvuI 4129
XhoI 4178
PvuII 4414
EcoRV 4709
SalI 4919
EcoRI 4931
PvuII 5186
NcoI 5512
EcoRI 5921
SmaI 5928

Figure 18

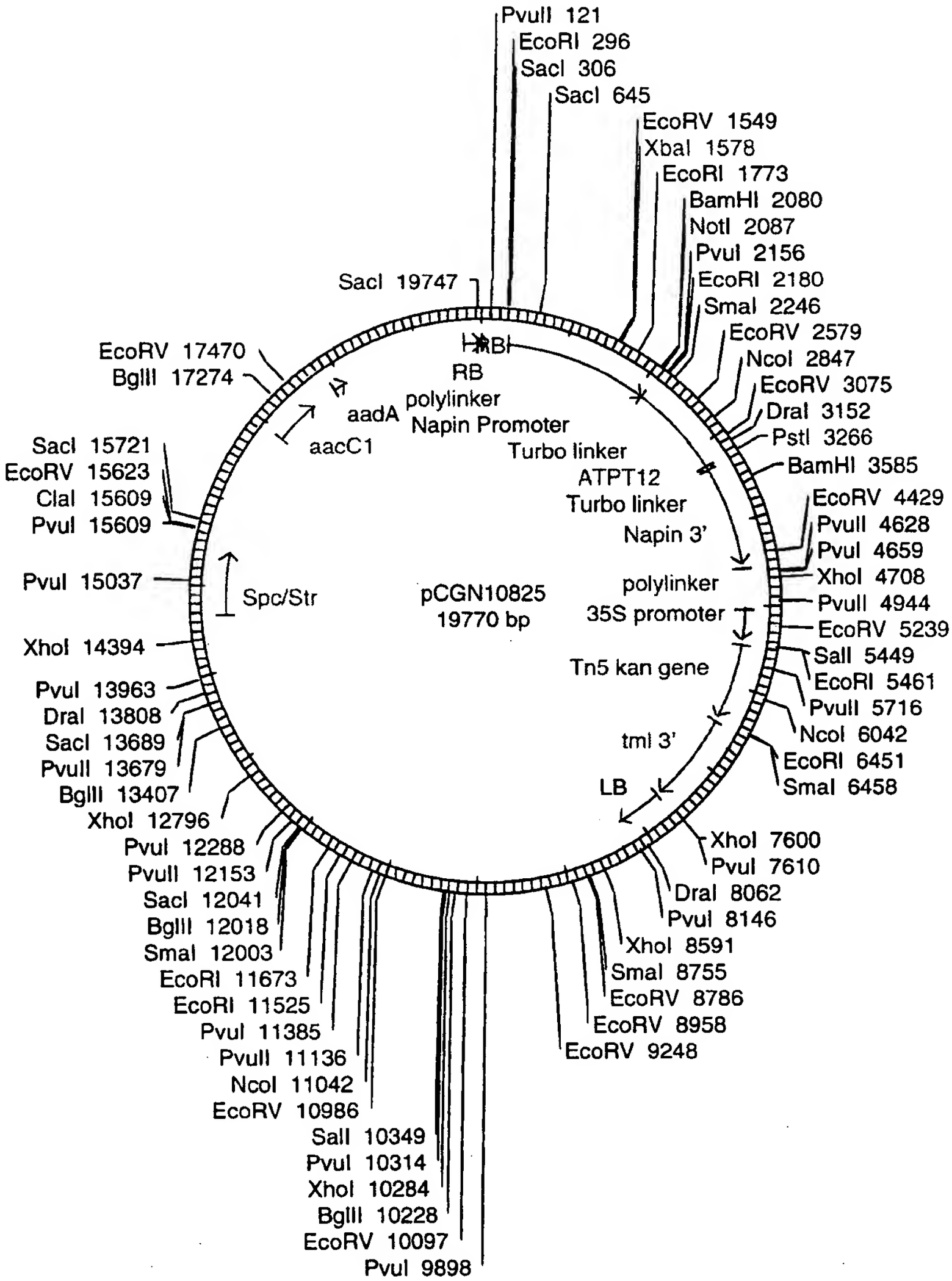
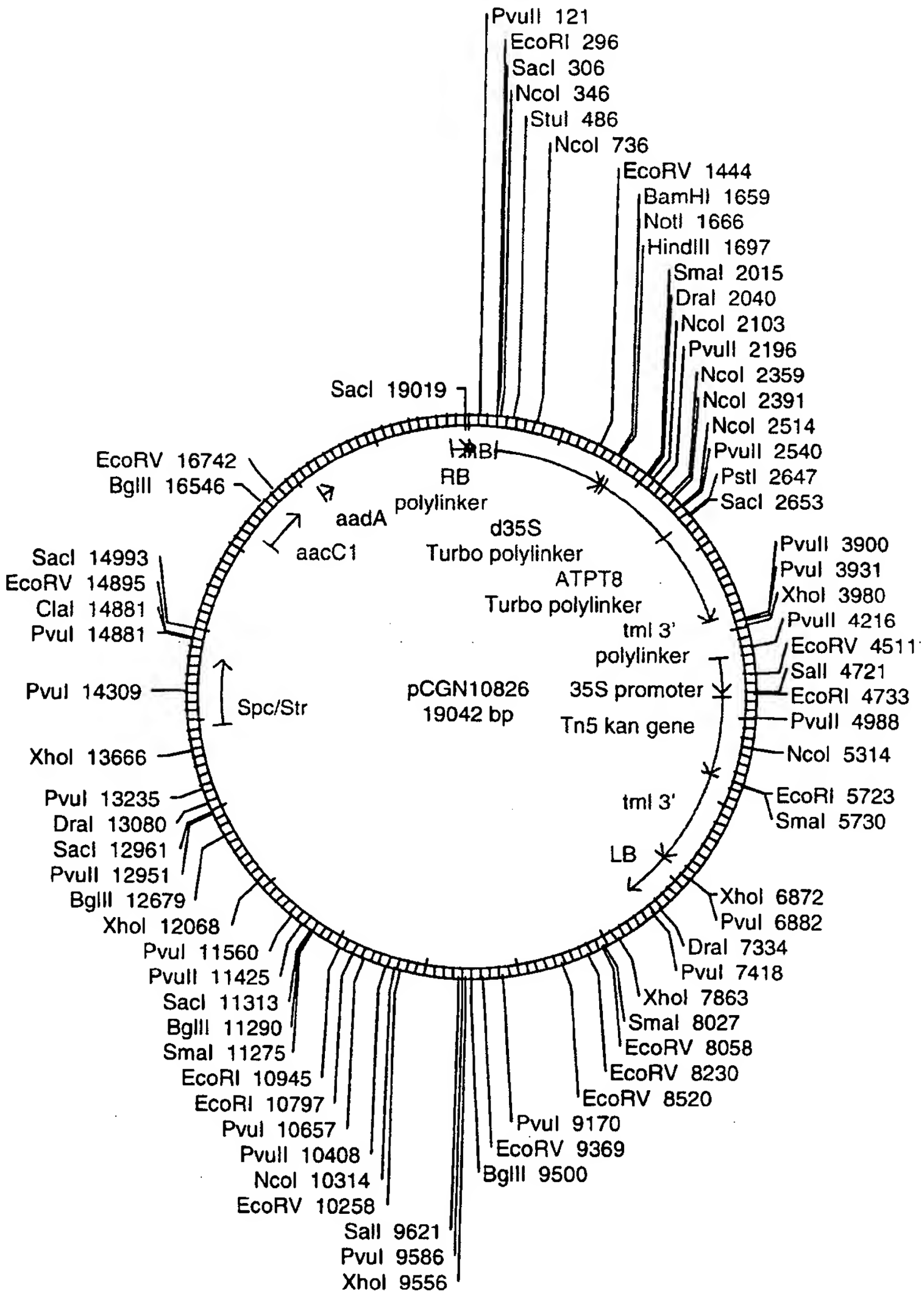

PvuII 121
EcoRI 296
SacI 306
SacI 645
EcoRV 1549
XbaI 1578
EcoRI 1773
BamHI 2080
NotI 2087
PvuI 2156
EcoRI 2180
SmaI 2246
EcoRV 2579
NcoI 2847
EcoRV 3075
DraI 3152
PstI 3266
BamHI 3585
EcoRV 4429
PvuII 4628
PvuI 4659
XhoI 4708
PvuII 4944
EcoRV 5239
SalI 5449
EcoRI 5461
PvuII 5716
NcoI 6042
EcoRI 6451
SmaI 6458
XhoI 7600
PvuI 7610
DraI 8062
PvuI 8146
XhoI 8591
SmaI 8755
EcoRV 8786
EcoRV 8958
EcoRV 9248
PvuI 9898
EcoRV 10097
BglII 10228
XhoI 10284
PvuI 10314
SalI 10349
NcoI 11042
PvuII 11136
PvuI 11385
EcoRI 11525
EcoRI 11673
SmaI 12003
BglII 12018
SacI 12041
PvuII 12153
PvuI 12288
XhoI 12796
BglII 13407
PvuII 13679
SacI 13689
DraI 13808
PvuI 13963
XhoI 14394
PvuI 15037
SacI 15721
EcoRV 15623
ClaI 15609
PvuI 15609
EcoRV 17470
BglII 17274
SacI 19747
RB
polylinker
Napin Promoter
Turbo linker
ATPT12
Turbo linker
Napin 3'
polylinker
35S promoter
Tn5 kan gene
tml 3'
LB
aadA
aacC1
Spc/Str
pCGN10825
19770 bp

Figure 19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35S promoter
Tn5 kan gene
tml 3'
LB
aadA
aacC1
Spc/Str
PvuII 121
EcoRI 296
SacI 306
NcoI 346
StuI 486
NcoI 736
EcoRV 1444
BamHI 1659
NotI 1666
HindIII 1697
SmaI 2015
DraI 2040
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PvuII 2196
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NcoI 2514
PvuII 2540
PstI 2647
SacI 2653
PvuII 3900
PvuI 3931
XhoI 3980
PvuII 4216
EcoRV 4511
SalI 4721
EcoRI 4733
PvuII 4988
NcoI 5314
EcoRI 5723
SmaI 5730
XhoI 6872
PvuI 6882
DraI 7334
PvuI 7418
XhoI 7863
SmaI 8027
EcoRV 8058
EcoRV 8230
EcoRV 8520
PvuI 9170
EcoRV 9369
BglII 9500
SalI 9621
PvuI 9586
XhoI 9556
XhoI 12068
PvuI 11560
PvuII 11425
SacI 11313
BglII 11290
SmaI 11275
EcoRI 10945
EcoRI 10797
PvuI 10657
PvuII 10408
NcoI 10314
EcoRV 10258
BglII 12679
PvuII 12951
SacI 12961
DraI 13080
PvuI 13235
XhoI 13666
PvuI 14309
SacI 14993
EcoRV 14895
ClaI 14881
PvuI 14881
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BglII 16546
SacI 19019

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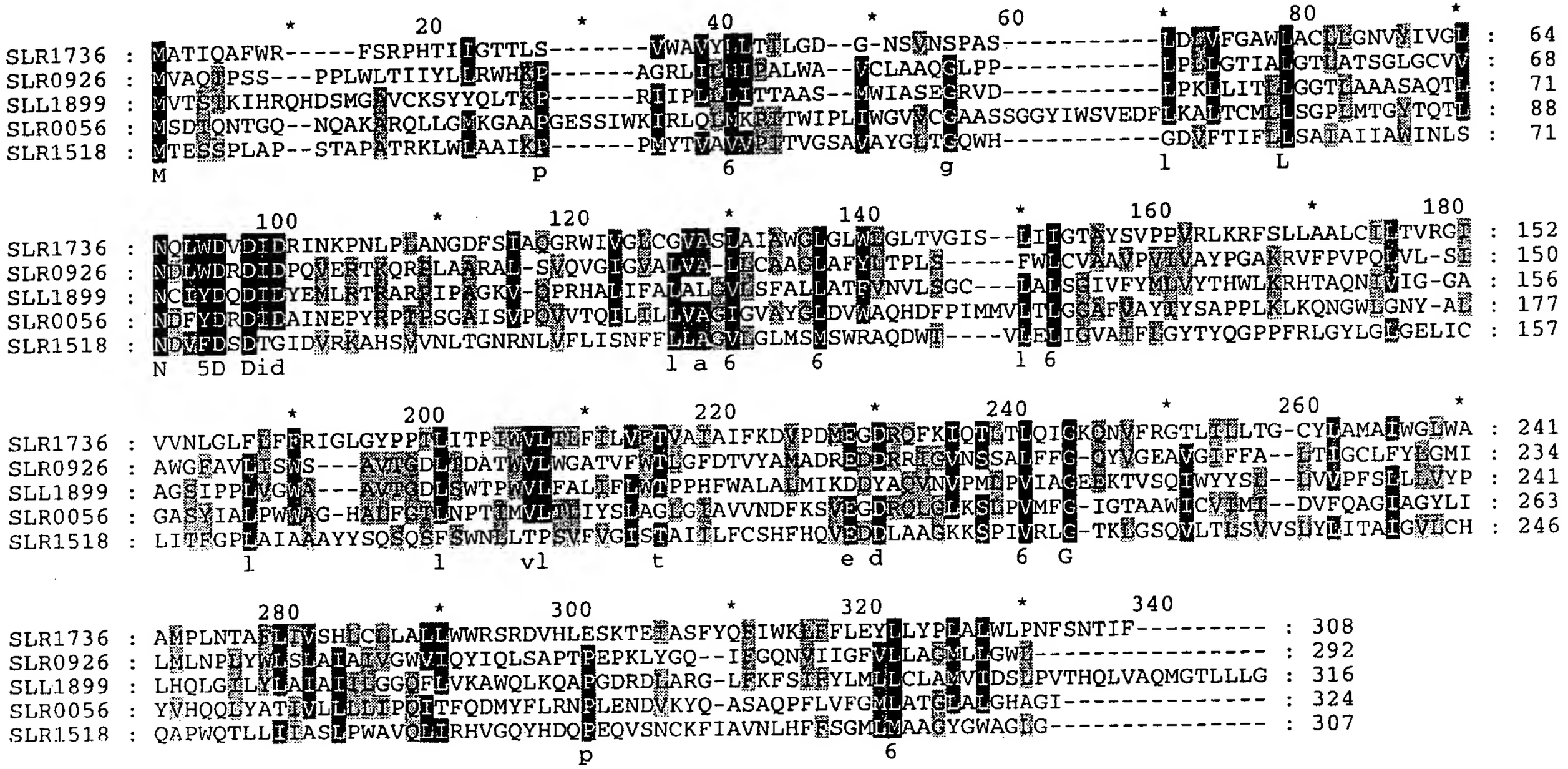
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SLL1899 : MVTSIKIHRQHDSMGFVCKSYYQLTKP------RIIPLFLITTAAS--MWIASEGRVD----------LPKLLITLLGGTLAAASAQTL : 71
SLR0056 : MSDTQNTGQ--NQAKTRQLLGMKGAAPGESSIWKIRLQLMKPFTWIPLIWGVVCGAASSGGYIWSVEDFLKALTCMLLSGPLMTGYTQTL : 88
SLR1518 : MTESSPLAP--STAPATRKLWLAAIKP------PMYTVAVVPITTVGSAVAYGLTGQWH----------GDVFTIFLLSAIAIIAWINLS : 71
M p 6 g l L
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SLR0926 : NDLWDRDIDPQVERTKQRPLAARALSSQVGIGVALVA-LLCAAGLAFYLTPLS-----FWLCVAAVPVIVAYPGAKRVFPVPQLVL-SI : 150
SLL1899 : NCLYDQDIDYEMLRTRARPIPAGKV-DPRHALIFALALCVLSFALLATPVNVLSGC---LALSCIVFYMLVYTHWLKRHTAQNIVIG-GA : 156
SLR0056 : NDFYDRDIDAINEPYRPTPSGAISVPQVVTQILILLVAGIGVAYGLDVWAQHDFPIMMVLTLGCAFVAYIYSAPPLKLKQNGWLGNY-AL : 177
SLR1518 : NDVFDSDTGIDVRKAHSVVNLTGNRNLVFLISNFFLLAGVLGLMSMSWRAQDWI----VLELIGVAIFIGYTYQGPPFRLGYLGLGELIC : 157
N 5D Did l a 6 6 l 6
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SLL1899 : AGSIPPLVGWA---AVTGDLSWTPWVLFALIFLWTPPHFWALAIMIKDDYAQVNVPMLPVIAGEEKTVSQIWYYSI--IVVPFSLLVYP : 241
SLR0056 : GASYIALPWWAG-HALFCTLNPTTMVLTLIYSLAGLGIAVVNDFKSVEGDRQLGLKSLPVMFG-IGTAAWICVIMI--DVFQAGIAGYLI : 263
SLR1518 : LITFGPLAIAAAYYSQSQSFSWNELTPSVFVGISTAILLFCSHFHQVEDDLAAGKKSPIVRLG-TKLGSQVLTLSVVSIYLITAIGVLCH : 246
l l vl t e d 6 G
SLR1736 : AMPLNTAFLIVSHLCLLALLWWRSRDVHLESKTEIASFYQFIWKLFFLEYLLYPLALWLPNFSNTIF---------- : 308
SLR0926 : LMLNPLYWLSLAIAIVGWVIQYIQLSAPTPEPKLYGQ--IFGQNVIIGFVLLAQMLLGWI---------------- : 292
SLL1899 : LHQLGILVLATAIIIGGQFLVKAWQLKQAPGDRDLARG-LFKFSIFYLMLLCLAMVIDSLPVTHQLVAQMGTLLLG : 316
SLR0056 : YVHQQLYATIVLLLLIPQITFQDMYFLRNPLENDVKYQ-ASAQPFLVFGMLATGLALGHAGI-------------- : 324
SLR1518 : QAPWQTLLIIASLPWAVQLIRHVGQYHDQPEQVSNCKFIAVNLHFFSGMLMAAGYGWAGLG-------------- : 307
p 6

Figure 21

```
                    *         20         *         40         *         60         *         80
ATPT2    : ---------MESLLSSSSLVSAAGGFCWKKQNLKLHSLSEIRVLRCDSSKVVAKPKFRNNLVRPDGQGSSLLLYPKHKSRFRVNATAGQ :  80
SLR1736  : ------------------------------------------------------------------------------------- :   -
ATPT3    : MAFFGLSRVSRRLLKSSVSVTPSSSSALLQSQHKSLSNPVTTHYTNPFTKCYPSWNDNYQVWSKGRELHQEKFFGVGWNYRLICGMSSS :  89
SLR0926  : ------------------------------------------------------------------------------------- :   -
ATPT4    : ------MWRRSVVYRFSSRISVSSSLPNPRLIPWSRELCAVNSFSQP----------------PVSTESTAKLGITGVRSDANRVFATA :  67
SLL1899  : -------------------------------------------------------------------------------------MVTS :   4
ATPT12   : ---------MTSILNTVSTIHSSRVTSVDRVGVLSLRNSDSVEFT----------------RRRSGFSTLIYESPGRRFVVRAAETDT :  63
SLR0056  : -------------------------------------------------------------------------------------MSDT :   4
ATPT8    : -------------------------------------------------------------------------------------MVLA :   4
SLR1518  : -------------------------------------------------------------------------------------MTES :   4

                *       100         *       120         *       140         *       160         *       1
ATPT2    : PEAFDSNSKQK----------SFRDSLDAFYR----------FSRPHTVIGTVLSILS-----VSFLAVEKVS--DISPLLFTGILE : 140
SLR1736  : ----------------------MATIQAFWR----------FSRPHTIIGTTLSVWAVY----LLTILGDGN-SVNSPASIDLVFG :  49
ATPT3    : SSVLEGKPKKDDKEKSDGVVVKKASWIDLYLPEEVRGYAKLARLDKPIGTWLLAWPCMWS------IALAADPGS--LPSFKYMALFGC : 170
SLR0926  : --MVAQTPSSP----------PLWLTIIYL---------LRWHKPAGRIILMIPALWA------VCLAAQ--G--LPPLPLLGTIAL :  56
ATPT4    : TAAATATATTG----EISSRVAALAGLGHHYAR--------CYWELSKAKLSMLVVATSG------TGYILGTGNAAISFPGLCYTCAG : 138
SLL1899  : TKIHRQHDSMG---------AVCKSYYQLTKP----------RIIPLLLITTAASMWI----------ASEGR--VDLPKLIITILG :  60
ATPT12   : DKVKSQTPDKAP---AGGSSINQLLGIKGAS----------QETNKWKIRLQLTKPVTWPPLVWGVVCGAAASGNFHWTPEDVAKSILC : 139
SLR0056  : QNT-GQNQAKA----------RQLLGMKGAAP---------GESSIWKIRLQLMKPITWIPLIWGVVCGAASSGGYIWSVEDFLKALTC :  73
ATPT8    : EVPKLASAAEY----------FFKRGVQGKQF----------RSTILLLMATALNVRVP------EALIGEST--DIVTSELRVRQR :  63
SLR1518  : SPLAPSTAPAT----------RKLWLAAIKP----------PMYTVAVVPITVG------SAVAYGLTG--QWHGDVFTIFLL :  59

                80          *         200         *         220         *         240         *         260
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SLR1736  : AWLACLLGNVYIVGLNQLWDVDIDRINKPNLPLANGDFSIAQGRWIVGLCGVASLAIAWGLG-LWLGLTVGLSLLIGTAY---SVPPVR : 134
ATPT3    : GALL---LRGAGCTINDLLDQDIDTKVDRTKLRPIASGLLT-PFQGLGFLGLQLLLGLG-----ILLQLNNYSRVLGAS----SLLIVF : 246
SLR0926  : GTLA---TSGLGCVVNDLWDRDIDPQVERTKQRPLAARALS-VQVGLGVALVALLCAAG-----LAFYLTPLSFWLCVA----AVPVIV : 132
ATPT4    : TMMI----AASANSLNQIFEISNDSKMKRTMLRPLPSGRISVPHAVAWATLAGASGACL-----LASKTNMLAAGLASAN---LVLYAF : 215
SLL1899  : GTLA----AASAQTLNCIYDQDIDYEMLETRARPIPAGKVQPRHALIFALALGVLSFAL-----LATFVNVLSGCLALSG---IVFYML : 137
ATPT12   : MMMSGPCLTGYTQTLNDWYDRDIDAINEPYRPIPSGAISEPEVITQVWVLLLGGLGIAGILD-VWAGHTTPTVFYLALG----GSLLSY : 223
SLR0056  : MLLSGPLMTGYTQTLNDFKDRDIDAINEPYRPIPSGAISVPQVVTQILILLVAGIGVAYGLD-VWAQHDFPIMMVLTLG----GAFVAY : 157
ATPT8    : GIAE---LTEMIHVASLLHDDVLDDADTRRGVGSLNVVMGNKMSVLAGDFLLSRACGALAAL-----AALKNTEVVALLATAVEHLVTGETM : 144
SLR1518  : SALA---LIAWINLSNDVFDSDTGIDVRKAHSVVNLTGNRNLVFLISNFFLLAGVLGLMSMS--WRAQDWTVLELLIGVA-----IFLGY : 138
                          n   d   d                                                       6
```

Figure 22 1/2

```
                 *         280         *         300         *         320         *         340         *
ATPT2    : WKR-FALVAAMCILAVRAIIVQIAFYLH-IQTHVFGRPILFTRPLIFATAFMSFFSVVIALFKDIPDIEGDKI-FGIRSFSVTLG-QKR : 313
SLR1736  : LKR-FSLLAALCILTVRGIVMNLGLFLF-FRIGLGYPPTLITPIWV-YTLFILVFLVAIAIFKDVPDMEGDRQ-FKIQTLTLQIG-KQN : 218
ATPT3    : SYP-LMKRFTFWPQAFLGLTINWGALLG--WT--AVKGSIAPSIVLPIYLSGVCVTLVYDTIYAHQDKEDDVK-VGVKSTALRFG-DNT : 328
SLR0926  : AYP-GAKRVFPVPQLVLSIAWGFAVLGS--WS--AVTGDLTDATWV-LWGATVFWTLGFDTVYAMADREDDRR-IGVNSSALFFG-QYV : 213
ATPT4    : VYT-PLKQLHPINTWVGAVVGAIPPLLG--WA--AASGQISYNSMI-IPAALYFWQIPHFMALAHLCRNDYAA-GGYKMLSLFDP---S : 294
SLL1899  : VYTHWLKRHTAQNIVIGGAAGSIPPLVG--WA--AVTGDLSWTPWV-IFALIFLWTPPHFWALALMIKDDYAQ-VNVPMLPVIAGEEKT : 220
ATPT12   : IYS-APPLKLKQNGWVGNFALGASYISLPWWAGQALFGTLIPDVVV-GTLLYSIAGLGIAIVNDFKSVEGDRA-LGLQSLPVAFG-TET : 308
SLR0056  : IYS-APPLKLKQNGWLGNYALGASYIALPWWAGHALFGTLNPTTMV-ITLIYSLAGLGIAVVNDFKSVEGDRQ-LGDKSLPVMFG-IGT : 242
ATPT8    : EITSSTEQRYSMDYYMQKTYYKTASLISNSCKAVAVLTGQIAEVAVLAFEYGRNLGLAFQLIDDILDFTGTSASLGKGSNSDIRH--GV : 231
SLR1518  : TYQGPPFRLGYLGLGELICLITFGPLAI-AAAYYSQSQSFSWNLLT-PSVFVGISTAIILFCSHFHQVEDDLA-AGKKSPIVRLG-TKL : 223
                                                                                    g         g

                  360         *         380         *         400         *         420         *         440
ATPT2    : VFWTCVTLLQMAYAVAILVGATSPFIWSKVISVVGHVILATTLWARAKSVDLSSKTEITS---CYMFIWKLFYAEYLLLPFLK------ : 393
SLR1736  : VFRGTLILLTGCYLAMALWGLWAAMPLNTAFLIVSHLCLLALLWWRSRDVHLESKTEIAS---FYQFIWKLFFLEYLLYPLALWLPNFS : 304
ATPT3    : KLWLTGFGTASIGFFALSGFSADLGWQYYASLAAAASGQLGWQIGTADLSSGADCS---------RKFVSNKWFGALIFSGVVLGRSFQ- : 407
SLR0926  : GEAVGIFFALTIGCLFYLGMILMLNPLYWLSLATAI--VGWVIQYIQLSAPTPEP-KLIY----GQIFGQNVIIGFVLLIAGMLLGWL--- : 292
ATPT4    : GKRIAAVALRNCFYMIPLGFLAYDWGLTSSWFCLESTLLTLAIAATAFSFYRDRTMHKA----RKMFHASLLFLPVFMSGLLLHRVSND : 379
SLL1899  : VSQIWYYSLLVVPFSLLIVYPLHQLGILYLALATIL--GGQFLVKAWQLKQAPGDRDIA----RGLFKFSIFYLMLLCLAMVIDSLPVT : 303
ATPT12   : AKWICVGAIDITQLSVAGYLIASGKPYYALALVALI--IPQIVFQFKYFLKDPVKYDVK----YQASAQPFLVLGIFVTALASQH---- : 387
SLR0056  : AAWICVIMIDVFQAGIAGYLIYVHQQLYATIVLLLL--IPQITFQDMYFLRNPLENDVK----YQASAQPFLVFGMLATGLAIGHAGI- : 324
ATPT8    : ITAPILFAMEEFPQIREVVDQVEKDPRNVDIALEYLGKSKGSQRARELAMEHANLAAAAIGSLPETDNEDVKRSRRALIDLTHRVITRN : 320
SLR1518  : GSQVLTLSVVSLYLITAIGVLCHQAPWQTLLIIASLPWAVQLIRHVGQYHDQPEQVSNCK---FIAVNLHFFSGMLMAAGYGWAGLG-- : 307

                 *         460         *         480         *
ATPT2    : ------------------------------------------------- :   -
SLR1736  : NTIF--------------------------------------------- : 308
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SLR0926  : ------------------------------------------------- :   -
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SLL1899  : --HQLVAQMGTLLLG---------------------------------- : 316
ATPT12   : ------------------------------------------------- :   -
SLR0056  : ------------------------------------------------- :   -
ATPT8    : K---------------------------------------------------- : 321
SLR1518  : ------------------------------------------------- :   -
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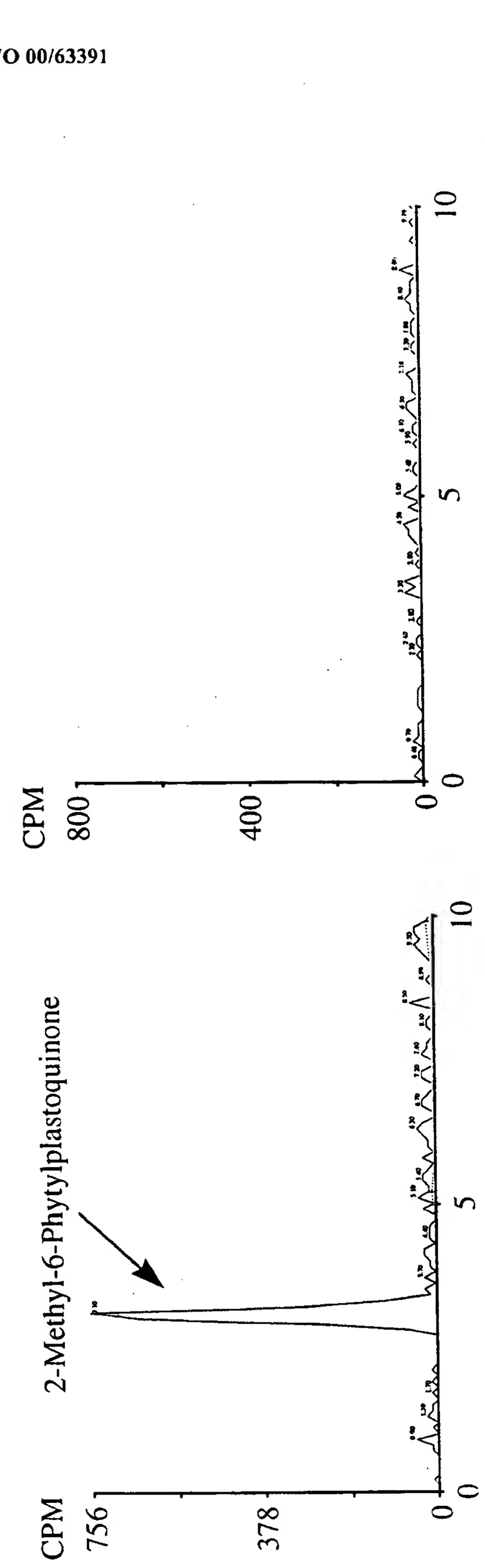
Figure 22 2/2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CPM
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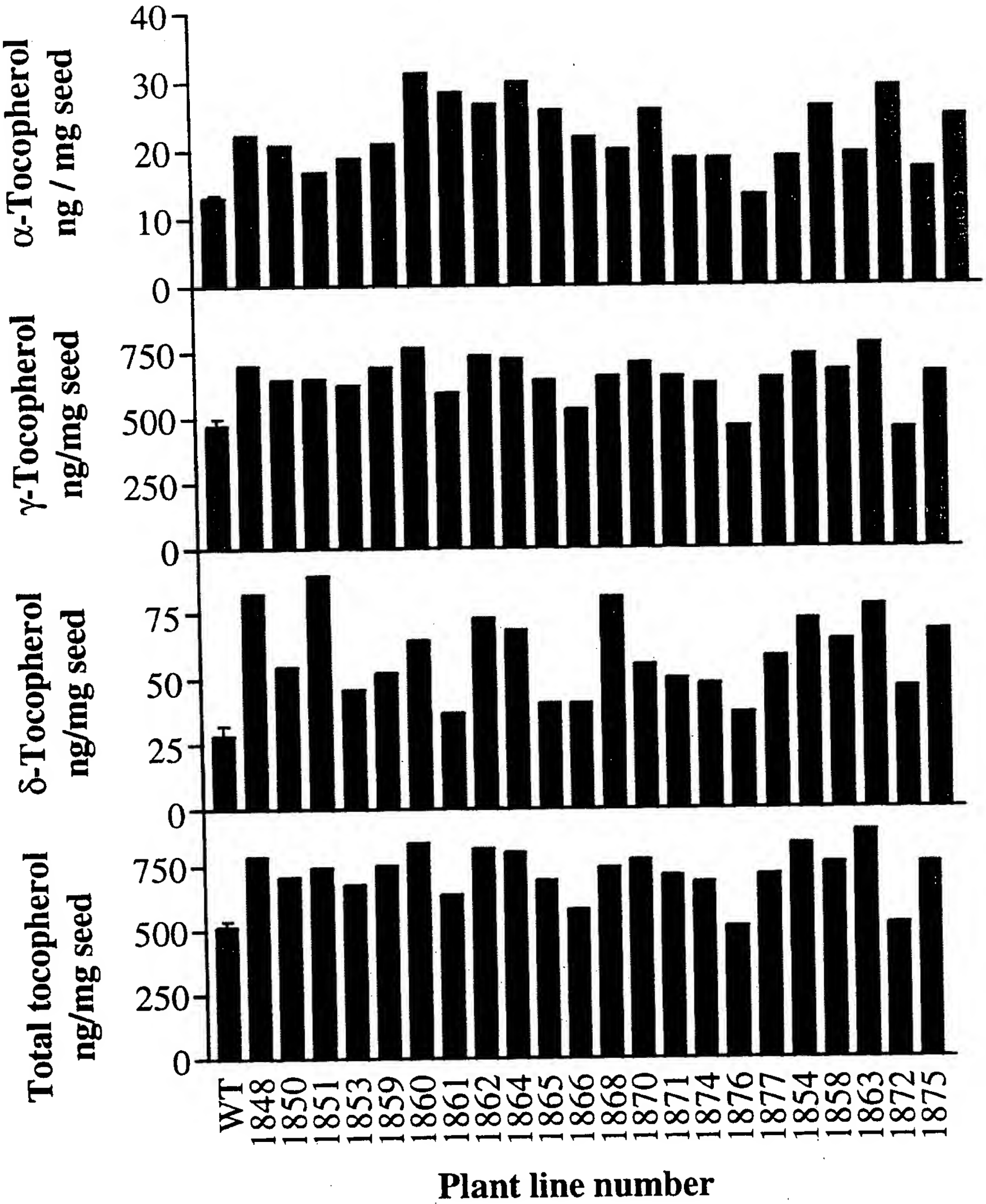
400
0
0
5
10
Time [min]
Synechocystis 6803 wild type
CPM
756
378
0
0
5
10
Time [min]
Synechocystis slr1736 knockout

Figure 23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40
30
20
10
0
γ-Tocopherol ng/mg seed
750
500
250
0
δ-Tocopherol ng/mg seed
75
50
25
0
Total tocopherol ng/mg seed
750
500
250
0
WT
1848
1850
1851
1853
1859
1860
1861
1862
1864
1865
1866
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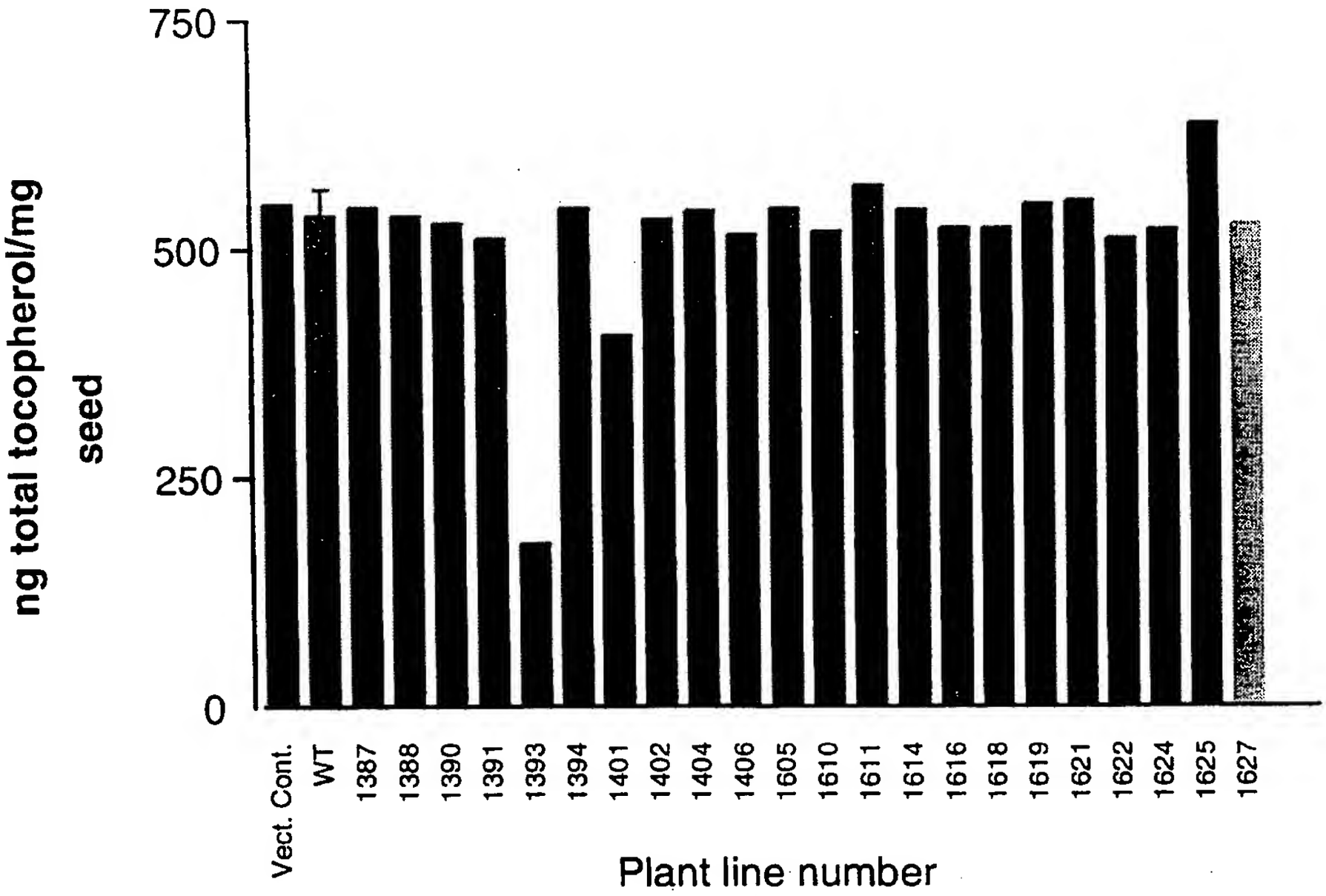
1874
1876
1877
1854
1858
1863
1872
1875
Plant line number

Figure 24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500
250
0
ng total tocopherol/mg seed
Vect. Cont.
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1388
1390
1391
1393
1394
1401
1402
1404
1406
1605
1610
1611
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1616
1618
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1621
1622
1624
1625
1627
Plant line number

Figure 25

SEQUENCE LISTING

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Arg Val Leu Arg Cys Asp Ser Ser Lys Val Val Ala Lys Pro Lys Phe
        35                  40                  45
Arg Asn Asn Leu Val Arg Pro Asp Gly Gln Gly Ser Ser Leu Leu Leu
    50                  55                  60
Tyr Pro Lys His Lys Ser Arg Phe Arg Val Asn Ala Thr Ala Gly Gln
65                  70                  75                  80
Pro Glu Ala Phe Asp Ser Asn Ser Lys Gln Lys Ser Phe Arg Asp Ser
                85                  90                  95
Leu Asp Ala Phe Tyr Arg Phe Ser Arg Pro His Thr Val Ile Gly Thr
            100                 105                 110
Val Leu Ser Ile Leu Ser Val Ser Phe Leu Ala Val Glu Lys Val Ser
        115                 120                 125
Asp Ile Ser Pro Leu Leu Phe Thr Gly Ile Leu Glu Ala Val Val Ala
    130                 135                 140
Ala Leu Met Met Asn Ile Tyr Ile Val Gly Leu Asn Gln Leu Ser Asp
145                 150                 155                 160
Val Glu Ile Asp Lys Val Asn Lys Pro Tyr Leu Pro Leu Ala Ser Gly
                165                 170                 175
Glu Tyr Ser Val Asn Thr Gly Ile Ala Ile Val Ala Ser Phe Ser Ile
            180                 185                 190
Met Ser Phe Trp Leu Gly Trp Ile Val Gly Ser Trp Pro Leu Phe Trp
        195                 200                 205
Ala Leu Phe Val Ser Phe Met Leu Gly Thr Ala Tyr Ser Ile Asn Leu
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Pro Leu Leu Arg Trp Lys Arg Phe Ala Leu Val Ala Ala Met Cys Ile
225                 230                 235                 240
Leu Ala Val Arg Ala Ile Ile Val Gln Ile Ala Phe Tyr Leu His Ile
                245                 250                 255
Gln Thr His Val Phe Gly Arg Pro Ile Leu Phe Thr Arg Pro Leu Ile
            260                 265                 270
Phe Ala Thr Ala Phe Met Ser Phe Phe Ser Val Val Ile Ala Leu Phe
        275                 280                 285
Lys Asp Ile Pro Asp Ile Glu Gly Asp Lys Ile Phe Gly Ile Arg Ser
    290                 295                 300
Phe Ser Val Thr Leu Gly Gln Lys Arg Val Phe Trp Thr Cys Val Thr
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Leu Leu Gln Met Ala Tyr Ala Val Ala Ile Leu Val Gly Ala Thr Ser
325 330 335
Pro Phe Ile Trp Ser Lys Val Ile Ser Val Val Gly His Val Ile Leu
340 345 350
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<400> 4

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Thr Lys Cys Tyr Pro Ser Trp Asn Asp Asn Tyr Gln Val Trp Ser Lys
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Gly Arg Glu Leu His Gln Glu Lys Phe Phe Gly Val Gly Trp Asn Tyr
65                  70                  75                  80
Arg Leu Ile Cys Gly Met Ser Ser Ser Ser Ser Val Leu Glu Gly Lys
                85                  90                  95
Pro Lys Lys Asp Asp Lys Glu Lys Ser Asp Gly Val Val Val Lys Lys
            100                 105                 110
Ala Ser Trp Ile Asp Leu Tyr Leu Pro Glu Glu Val Arg Gly Tyr Ala
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Lys Leu Ala Arg Leu Asp Lys Pro Ile Gly Thr Trp Leu Leu Ala Trp
    130                 135                 140
Pro Cys Met Trp Ser Ile Ala Leu Ala Ala Asp Pro Gly Ser Leu Pro
145                 150                 155                 160
Ser Phe Lys Tyr Met Ala Leu Phe Gly Cys Gly Ala Leu Leu Leu Arg
                165                 170                 175
Gly Ala Gly Cys Thr Ile Asn Asp Leu Leu Asp Gln Asp Ile Asp Thr
            180                 185                 190
Lys Val Asp Arg Thr Lys Leu Arg Pro Ile Ala Ser Gly Leu Leu Thr
        195                 200                 205
Pro Phe Gln Gly Ile Gly Phe Leu Gly Leu Gln Leu Leu Leu Gly Leu
    210                 215                 220
Gly Ile Leu Leu Gln Leu Asn Asn Tyr Ser Arg Val Leu Gly Ala Ser
225                 230                 235                 240
Ser Leu Leu Leu Val Phe Ser Tyr Pro Leu Met Lys Arg Phe Thr Phe
                245                 250                 255
Trp Pro Gln Ala Phe Leu Gly Leu Thr Ile Asn Trp Gly Ala Leu Leu
            260                 265                 270
Gly Trp Thr Ala Val Lys Gly Ser Ile Ala Pro Ser Ile Val Leu Pro
        275                 280                 285
Leu Tyr Leu Ser Gly Val Cys Trp Thr Leu Val Tyr Asp Thr Ile Tyr
    290                 295                 300
Ala His Gln Asp Lys Glu Asp Asp Val Lys Val Gly Val Lys Ser Thr
305                 310                 315                 320
Ala Leu Arg Phe Gly Asp Asn Thr Lys Leu Trp Leu Thr Gly Phe Gly
                325                 330                 335
Thr Ala Ser Ile Gly Phe Leu Ala Leu Ser Gly Phe Ser Ala Asp Leu
            340                 345                 350
Gly Trp Gln Tyr Tyr Ala Ser Leu Ala Ala Ala Ser Gly Gln Leu Gly
        355                 360                 365
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Trp Gln Ile Gly Thr Ala Asp Leu Ser Ser Gly Ala Asp Cys Ser Arg
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Lys Phe Val Ser Asn Lys Trp Phe Gly Ala Ile Ile Phe Ser Gly Val
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Val Leu Gly Arg Ser Phe Gln
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<212> PRT
<213> Arabidopsis sp

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35 40 45
Ala Lys Leu Gly Ile Thr Gly Val Arg Ser Asp Ala Asn Arg Val Phe
50 55 60
Ala Thr Ala Thr Ala Ala Ala Thr Ala Thr Ala Thr Thr Gly Glu Ile
65 70 75 80
Ser Ser Arg Val Ala Ala Leu Ala Gly Leu Gly His His Tyr Ala Arg
85 90 95
Cys Tyr Trp Glu Leu Ser Lys Ala Lys Leu Ser Met Leu Val Val Ala
100 105 110
Thr Ser Gly Thr Gly Tyr Ile Leu Gly Thr Gly Asn Ala Ala Ile Ser
115 120 125
Phe Pro Gly Leu Cys Tyr Thr Cys Ala Gly Thr Met Met Ile Ala Ala
130 135 140
Ser Ala Asn Ser Leu Asn Gln Ile Phe Glu Ile Ser Asn Asp Ser Lys
145 150 155 160
Met Lys Arg Thr Met Leu Arg Pro Leu Pro Ser Gly Arg Ile Ser Val
165 170 175
Pro His Ala Val Ala Trp Ala Thr Ile Ala Gly Ala Ser Gly Ala Cys
180 185 190
Leu Leu Ala Ser Lys Thr Asn Met Leu Ala Ala Gly Leu Ala Ser Ala
195 200 205
Asn Leu Val Leu Tyr Ala Phe Val Tyr Thr Pro Leu Lys Gln Leu His
210 215 220
Pro Ile Asn Thr Trp Val Gly Ala Val Val Gly Ala Ile Pro Pro Leu
225 230 235 240
Leu Gly Trp Ala Ala Ala Ser Gly Gln Ile Ser Tyr Asn Ser Met Ile
245 250 255
Leu Pro Ala Ala Leu Tyr Phe Trp Gln Ile Pro His Phe Met Ala Leu
260 265 270
Ala His Leu Cys Arg Asn Asp Tyr Ala Ala Gly Gly Tyr Lys Met Leu
275 280 285
Ser Leu Phe Asp Pro Ser Gly Lys Arg Ile Ala Ala Val Ala Leu Arg
290 295 300
Asn Cys Phe Tyr Met Ile Pro Leu Gly Phe Ile Ala Tyr Asp Trp Gly
305 310 315 320
Leu Thr Ser Ser Trp Phe Cys Leu Glu Ser Thr Leu Leu Thr Leu Ala
325 330 335
Ile Ala Ala Thr Ala Phe Ser Phe Tyr Arg Asp Arg Thr Met His Lys
340 345 350
Ala Arg Lys Met Phe His Ala Ser Leu Leu Phe Leu Pro Val Phe Met
355 360 365
Ser Gly Leu Leu Leu His Arg Val Ser Asn Asp Asn Gln Gln Gln Leu
370 375 380
Val Glu Glu Ala Gly Leu Thr Asn Ser Val Ser Gly Glu Val Lys Thr
385 390 395 400

Gln Arg Arg Lys Lys Arg Val Ala Gln Pro Pro Val Ala Tyr Ala Ser
405 410 415
Ala Ala Pro Phe Pro Phe Leu Pro Ala Pro Ser Phe Tyr Ser Pro
420 425 430

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<212> DNA
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<212> PRT
<213> Arabidopsis sp

<400> 9

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Leu Met Ala Thr Ala Leu Asn Val Arg Val Pro Glu Ala Leu Ile Gly
35 40 45

Glu Ser Thr Asp Ile Val Thr Ser Glu Leu Arg Val Arg Gln Arg Gly
50 55 60

Ile Ala Glu Ile Thr Glu Met Ile His Val Ala Ser Leu Leu His Asp
65 70 75 80

Asp Val Leu Asp Asp Ala Asp Thr Arg Arg Gly Val Gly Ser Leu Asn
85 90 95

Val Val Met Gly Asn Lys Val Val Ala Leu Leu Ala Thr Ala Val Glu
100 105 110

His Leu Val Thr Gly Glu Thr Met Glu Ile Thr Ser Ser Thr Glu Gln
115 120 125

Arg Tyr Ser Met Asp Tyr Tyr Met Gln Lys Thr Tyr Tyr Lys Thr Ala
130 135 140

Ser Leu Ile Ser Asn Ser Cys Lys Ala Val Ala Val Leu Thr Gly Gln
145 150 155 160

Thr Ala Glu Val Ala Val Leu Ala Phe Glu Tyr Gly Arg Asn Leu Gly
165 170 175

Leu Ala Phe Gln Leu Ile Asp Asp Ile Leu Asp Phe Thr Gly Thr Ser
180 185 190

Ala Ser Leu Gly Lys Gly Ser Leu Ser Asp Ile Arg His Gly Val Ile
195 200 205

Thr Ala Pro Ile Leu Phe Ala Met Glu Glu Phe Pro Gln Leu Arg Glu
210 215 220

Val Val Asp Gln Val Glu Lys Asp Pro Arg Asn Val Asp Ile Ala Leu
225 230 235 240

Glu Tyr Leu Gly Lys Ser Lys Gly Ile Gln Arg Ala Arg Glu Leu Ala
245 250 255

Met Glu His Ala Asn Leu Ala Ala Ala Ala Ile Gly Ser Leu Pro Glu
260 265 270

Thr Asp Asn Glu Asp Val Lys Arg Ser Arg Arg Ala Leu Ile Asp Leu
275 280 285

Thr His Arg Val Ile Thr Arg Asn Lys
290 295

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<211> 561

<212> DNA

<213> Arabidopsis sp

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<211> 966
<212> DNA
<213> Arabidopsis sp

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<212> PRT
<213> Arabidopsis sp

<400> 12
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35 40 45
Glu Ser Thr Asp Ile Val Thr Ser Glu Leu Arg Val Arg Gln Arg Gly
50 55 60
Ile Ala Glu Ile Thr Glu Met Ile His Val Ala Ser Leu Leu His Asp
65 70 75 80
Asp Val Leu Asp Asp Ala Asp Thr Arg Arg Gly Val Gly Ser Leu Asn
85 90 95
Val Val Met Gly Asn Lys Met Ser Val Leu Ala Gly Asp Phe Leu Leu
100 105 110
Ser Arg Ala Cys Gly Ala Leu Ala Ala Leu Lys Asn Thr Glu Val Val
115 120 125
Ala Leu Leu Ala Thr Ala Val Glu His Leu Val Thr Gly Glu Thr Met
130 135 140
Glu Ile Thr Ser Ser Thr Glu Gln Arg Tyr Ser Met Asp Tyr Tyr Met
145 150 155 160
Gln Lys Thr Tyr Tyr Lys Thr Ala Ser Leu Ile Ser Asn Ser Cys Lys
165 170 175
Ala Val Ala Val Leu Thr Gly Gln Thr Ala Glu Val Ala Val Leu Ala
180 185 190
Phe Glu Tyr Gly Arg Asn Leu Gly Leu Ala Phe Gln Leu Ile Asp Asp
195 200 205
Ile Leu Asp Phe Thr Gly Thr Ser Ala Ser Leu Gly Lys Gly Ser Leu
210 215 220
Ser Asp Ile Arg His Gly Val Ile Thr Ala Pro Ile Leu Phe Ala Met
225 230 235 240
Glu Glu Phe Pro Gln Leu Arg Glu Val Val Asp Gln Val Glu Lys Asp
245 250 255
Pro Arg Asn Val Asp Ile Ala Leu Glu Tyr Leu Gly Lys Ser Lys Gly
260 265 270
Ile Gln Arg Ala Arg Glu Leu Ala Met Glu His Ala Asn Leu Ala Ala
275 280 285
Ala Ala Ile Gly Ser Leu Pro Glu Thr Asp Asn Glu Asp Val Lys Arg
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Ser Arg Arg Ala Leu Ile Asp Leu Thr His Arg Val Ile Thr Arg Asn
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Lys

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<212> DNA
<213> Arabidopsis sp

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<211> 741
<212> DNA
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<211> 1087
<212> DNA
<213> Arabidopsis sp

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<211> 1164
<212> DNA
<213> Arabidopsis sp

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<210> 17
<211> 387
<212> PRT
<213> Arabidopsis sp

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Val Glu Phe Thr Arg Arg Arg Ser Gly Phe Ser Thr Leu Ile Tyr Glu
        35                  40                  45
Ser Pro Gly Arg Arg Phe Val Val Arg Ala Ala Glu Thr Asp Thr Asp
    50                  55                  60
Lys Val Lys Ser Gln Thr Pro Asp Lys Ala Pro Ala Gly Gly Ser Ser
65                  70                  75                  80
Ile Asn Gln Leu Leu Gly Ile Lys Gly Ala Ser Gln Glu Thr Asn Lys
                85                  90                  95
Trp Lys Ile Arg Leu Gln Leu Thr Lys Pro Val Thr Trp Pro Pro Leu
            100                 105                 110
Val Trp Gly Val Val Cys Gly Ala Ala Ala Ser Gly Asn Phe His Trp
        115                 120                 125
Thr Pro Glu Asp Val Ala Lys Ser Ile Leu Cys Met Met Met Ser Gly
    130                 135                 140
Pro Cys Leu Thr Gly Tyr Thr Gln Thr Ile Asn Asp Trp Tyr Asp Arg
145                 150                 155                 160
Asp Ile Asp Ala Ile Asn Glu Pro Tyr Arg Pro Ile Pro Ser Gly Ala
                165                 170                 175
Ile Ser Glu Pro Glu Val Ile Thr Gln Val Trp Val Leu Leu Leu Gly
            180                 185                 190
Gly Leu Gly Ile Ala Gly Ile Leu Asp Val Trp Ala Gly His Thr Thr
        195                 200                 205
Pro Thr Val Phe Tyr Leu Ala Leu Gly Gly Ser Leu Leu Ser Tyr Ile
    210                 215                 220
Tyr Ser Ala Pro Pro Leu Lys Leu Lys Gln Asn Gly Trp Val Gly Asn
225                 230                 235                 240
Phe Ala Leu Gly Ala Ser Tyr Ile Ser Leu Pro Trp Trp Ala Gly Gln
                245                 250                 255
Ala Leu Phe Gly Thr Leu Thr Pro Asp Val Val Val Leu Thr Leu Leu
            260                 265                 270
Tyr Ser Ile Ala Gly Leu Gly Ile Ala Ile Val Asn Asp Phe Lys Ser
        275                 280                 285
Val Glu Gly Asp Arg Ala Leu Gly Leu Gln Ser Leu Pro Val Ala Phe
    290                 295                 300
Gly Thr Glu Thr Ala Lys Trp Ile Cys Val Gly Ala Ile Asp Ile Thr
305                 310                 315                 320
Gln Leu Ser Val Ala Gly Tyr Leu Leu Ala Ser Gly Lys Pro Tyr Tyr
                325                 330                 335
Ala Leu Ala Leu Val Ala Leu Ile Ile Pro Gln Ile Val Phe Gln Phe
            340                 345                 350
Lys Tyr Phe Leu Lys Asp Pro Val Lys Tyr Asp Val Lys Tyr Gln Ala
        355                 360                 365
Ser Ala Gln Pro Phe Leu Val Leu Gly Ile Phe Val Thr Ala Leu Ala
    370                 375                 380
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Ser Gln His
385

<210> 18
<211> 981
<212> DNA
<213> Arabidopsis sp

<400> 18
atgttgttta gtggttcagc gatcccatta agcagcttct gctctcttcc ggagaaaccc 60
cacactcttc ctatgaaact ctctcccgct gcaatccgat cttcatcctc atctgccccg 120
gggtcgttga acttcgatct gaggacgtat tggacgactc tgatcaccga gatcaaccag 180
aagctggatg aggccatacc ggtcaagcac cctgcgggga tctacgaggc tatgagatac 240
tctgtactcg cacaaggcgc caagcgtgcc cctcctgtga tgtgtgtggc ggcctgcgag 300
ctcttcggtg gcgatcgcct cgccgctttc cccaccgcct gtgccctaga aatggtgcac 360
gcggcttcgt tgatacacga cgacctcccc tgtatggacg acgatcctgt gcgcagagga 420
aagccatcta accacactgt ctacggctct ggcatggcca ttctcgccgg tgacgccctc 480
ttcccactcg ccttccagca cattgtctcc cacacgcctc ctgaccttgt tccccgagcc 540
accatcctca gactcatcac tgagattgcc cgcactgtcg gctccactgg tatggctgca 600
ggccagtacg tcgaccttga aggaggtccc tttcctcttt cctttgttca ggagaagaaa 660
ttcggagcca tgggtgaatg ctctgccgtg tgcggtggcc tattgggcgg tgccactgag 720
gatgagctcc agagtctccg aaggtacggg agagccgtcg ggatgctgta tcaggtggtc 780
gatgacatca ccgaggacaa gaagaagagc tatgatggtg gagcagagaa gggaatgatg 840
gaaatggcgg aagagctcaa ggagaaggcg aagaaggagc ttcaagtgtt tgacaacaag 900
tatggaggag gagacacact tgttcctctc tacaccttcg ttgactacgc tgctcatcga 960
cattttcttc ttccctctg a 981

<210> 19
<211> 245
<212> DNA
<213> GLycine sp

<400> 19
gcaacatctg ggactgggtt tgtcttgggg agtggtagtg ctgttgatct ttcggcactt 60
tcttgcactt gcttgggtac catgatggtt gctgcatctg ctaactcttt gaatcaggtg 120
tttgagatca ataatgatgc taaaatgaag agaacaagtc gcaggccact accctcagga 180
cgcatcacaa tacctcatgc agttggctgg gcatcctctg ttggattagc tggtacggct 240
ctact 245

<210> 20
<211> 253
<212> DNA
<213> Glycine sp

<400> 20
attggctttc caagatcatt gggttttctt gttgcattca tgaccttcta ctccttgggt 60

ttggcattgt ccaaggatat acctgacgtt gaaggagata aagagcacgg cattgattct 120
tttgcagtac gtctaggtca gaaacgggca ttttggattt gcgtttcctt ttttgaaatg 180
gctttcggag ttggtatcct ggccggagca tcatgctcac acttttggac taaaattttc 240
acgggtatgg gaa 253

<210> 21
<211> 275
<212> DNA
<213> Glycine sp

<400> 21
tgatcttcta ctctctgggt atggcattgt ccaaggatat atctgacgtt aaaggagata 60
aagcatacgg catcgatact ttagcgatac gtttgggtca aaaatgggta ttttggattt 120
gcattatcct ttttgaaatg gcttttggag ttgccctctt ggcaggagca acatcttctt 180
acctttggat taaaattgtc acgggtctgg gacatgctat tcttgcttca attctcttgt 240
accaagccaa atctatatac ttgagcaaca aagtt 275

<210> 22
<211> 299
<212> DNA
<213> Glycine sp

<220>
<221> misc_feature
<222> (1)...(299)
<223> n = A,T,C or G

<400> 22
ccanaatang tncatcttng aaagacaatt ggcctcttca acacacaagt ctgcatgtga 60
agaagaggcc aattgtcttt ccaagatcac ttatngtggc tattgtaatc atgaacttct 120
tctttgtggg tatggcattg gcaaaggata tacctanctg ttgaaggaga taaaatatat 180
ggcattgata cttttgcaat acgtataggt caaaaacaag tattttggat ttgtattttc 240
ctttttgaaa ggctttcgga gtttccctag tggcaggagc aacatcttct agccttggt 299

<210> 23
<211> 767
<212> DNA
<213> Glycine sp

<400> 23
gtggaggctg tggttgctgc cctgtttatg aatatttata ttgttggttt gaatcaattg 60
tctgatgttg aaatagacaa gataaacaag ccgtatcttc cattagcatc tggggaatat 120
tcctttgaaa ctggtgtcac tattgttgca tctttttcaa ttctgagttt ttggcttggc 180
tgggttgtag gttcatggcc attatttttgg gccctttttg taagctttgt gctaggaact 240
gcttattcaa tcaatgtgcc tctgttgaga tggaagaggt ttgcagtgct tgcagcgatg 300
tgcattctag ctgttcgggc agtaatagtt caacttgcat ttttccttca catgcagact 360

catgtgtaca agaggccacc tgtcttttca agaccattga tttttgctac tgcattcatg 420
agcttcttct ctgtagttat agcactgttt aaggatatac ctgacattga aggagataaa 480
gtatttggca tccaatcttt ttcagtgtgt ttaggtcaga agccggtgtt ctggacttgt 540
gttacccttc ttgaaatagc ttatggagtc gccctcctgg tgggagctgc atctccttgt 600
ctttggagca aaattttcac gggtctggga cacgctgtgc tggcttcaat tctctggttt 660
catgccaaat ctgtagattt gaaaagcaaa gcttcgataa catccttcta tatgtttatt 720
tggaagctat tttatgcaga atacttactc attccttttg ttagatg 767

<210> 24
<211> 255
<212> PRT
<213> Glycine sp

<400> 24

Val Glu Ala Val Val Ala Ala Leu Phe Met Asn Ile Tyr Ile Val Gly
1 5 10 15
Leu Asn Gln Leu Ser Asp Val Glu Ile Asp Lys Ile Asn Lys Pro Tyr
20 25 30
Leu Pro Leu Ala Ser Gly Glu Tyr Ser Phe Glu Thr Gly Val Thr Ile
35 40 45
Val Ala Ser Phe Ser Ile Leu Ser Phe Trp Leu Gly Trp Val Val Gly
50 55 60
Ser Trp Pro Leu Phe Trp Ala Leu Phe Val Ser Phe Val Leu Gly Thr
65 70 75 80
Ala Tyr Ser Ile Asn Val Pro Leu Leu Arg Trp Lys Arg Phe Ala Val
85 90 95
Leu Ala Ala Met Cys Ile Leu Ala Val Arg Ala Val Ile Val Gln Leu
100 105 110
Ala Phe Phe Leu His Met Gln Thr His Val Tyr Lys Arg Pro Pro Val
115 120 125
Phe Ser Arg Pro Leu Ile Phe Ala Thr Ala Phe Met Ser Phe Phe Ser
130 135 140
Val Val Ile Ala Leu Phe Lys Asp Ile Pro Asp Ile Glu Gly Asp Lys
145 150 155 160
Val Phe Gly Ile Gln Ser Phe Ser Val Cys Leu Gly Gln Lys Pro Val
165 170 175
Phe Trp Thr Cys Val Thr Leu Leu Glu Ile Ala Tyr Gly Val Ala Leu
180 185 190
Leu Val Gly Ala Ala Ser Pro Cys Leu Trp Ser Lys Ile Phe Thr Gly
195 200 205
Leu Gly His Ala Val Leu Ala Ser Ile Leu Trp Phe His Ala Lys Ser
210 215 220
Val Asp Leu Lys Ser Lys Ala Ser Ile Thr Ser Phe Tyr Met Phe Ile
225 230 235 240
Trp Lys Leu Phe Tyr Ala Glu Tyr Leu Leu Ile Pro Phe Val Arg
245 250 255

<210> 25
<211> 360
<212> DNA
<213> Zea sp

<220>
<221> misc_feature
<222> (1)...(360)
<223> n = A,T,C or G

<400> 25
ggcgtcttca cttgttctgg tcttctcgta tcccctgatg aagaggttca cattttggcc 60
tcaggcttat cttggcctga cattcaactg gggagcttta ctagggtggg ctgctattaa 120
ggaaagcata gaccctgcaa atcatccttc cattgtatac agctggtatt tgttggacgc 180
tggtgtatga tactatatat gcgcatcagg tgtttcgcta tccctacttt catattaatc 240
cttgatgaag tggccatttc atgttgtcgc ggtggtctta tacttgcata tctccatgca 300
tctcaggaca aagangatga cctgaaagta ggagtccaag tccacagctt aagatttggg 360

<210> 26
<211> 299
<212> DNA
<213> Zea sp

<220>
<221> misc_feature
<222> (1)...(299)
<223> n = A,T,C or G

<400> 26
gatggttgca gcatctgcaa ataccctcaa ccaggtgttt gngataaaaa atgatgctaa 60
aatgaaaagg acaatgcgtg ccccctgcca tctggtcgca ttagtcctgc acatgctgcg 120
atgtgggcta caagtgttgg agttgcagga acagctttgt tggcctggaa ggctaatggc 180
ttggcagctg ggcttgcagc ttctaatctt gttctgtatg catttgtgta tacgccgttg 240
aagcaaatac accctgttaa tacatgggtt ggggcagtcg ttggtgccat cccaccact 299

<210> 27
<211> 255
<212> DNA
<213> Zea sp

<220>
<221> misc_feature
<222> (1)...(255)
<223> n = A,T,C or G

<400> 27
anacttgcat atctccatgc ntctcaggac aaagangatg acctgaaagt aggtgtcaag 60
tccacagcat taagatttgg agatttgacc nnatactgna tcagtggctt tggcgcggca 120
tgcttcggca gcttagcact cagtggttac aatgctgacc ttggttggtg tttagtgtga 180
tgcttgagcg aagaatggta tngtttttac ttgatattga ctccagacct gaaatcatgt 240
tggacagggt ggccc 255

<210> 28
<211> 257
<212> DNA
<213> Zea sp

<400> 28
attgaagggg ataggactct ggggcttcag tcacttcctg ttgcttttgg gatggaaact 60
gcaaaatgga tttgtgttgg agcaattgat atcactcaat tatctgttgc aggttaccta 120
ttgagcaccg gtaagctgta ttatgccctg gtgttgcttg ggctaacaat tcctcaggtg 180
ttctttcagt tccagtactt cctgaaggac cctgtgaagt atgatgtcaa atatcaggca 240
agcgcacaac cattctt 257

<210> 29
<211> 368
<212> DNA
<213> Zea sp

<400> 29
atccagttgc aaataataat ggcgttcttc tctgttgtaa tagcactatt caaggatata 60
cctgacatcg aaggggaccg catattcggg atccgatcct tcagcgtccg gttagggcaa 120
aagaaggtct tttggatctg cgttggcttg cttgagatgg cctacagcgt tgcgatactg 180
atgggagcta cctcttcctg tttgtggagc aaaacagcaa ccatcgctgg ccattccata 240
cttgccgcga tcctatggag ctgcgcgcga tcggtggact tgacgagcaa agccgcaata 300
acgtccttct acatgttcat ctggaagctg ttctacgcgg agtacctgct catccctctg 360
gtgcggtg 368

<210> 30
<211> 122
<212> PRT
<213> Zea sp

<400> 30
Ile Gln Leu Gln Ile Ile Met Ala Phe Phe Ser Val Val Ile Ala Leu
1 5 10 15
Phe Lys Asp Ile Pro Asp Ile Glu Gly Asp Arg Ile Phe Gly Ile Arg
20 25 30
Ser Phe Ser Val Arg Leu Gly Gln Lys Lys Val Phe Trp Ile Cys Val
35 40 45
Gly Leu Leu Glu Met Ala Tyr Ser Val Ala Ile Leu Met Gly Ala Thr

50 55 60

Ser Ser Cys Leu Trp Ser Lys Thr Ala Thr Ile Ala Gly His Ser Ile
65 70 75 80
Leu Ala Ala Ile Leu Trp Ser Cys Ala Arg Ser Val Asp Leu Thr Ser
85 90 95
Lys Ala Ala Ile Thr Ser Phe Tyr Met Phe Ile Trp Lys Leu Phe Tyr
100 105 110
Ala Glu Tyr Leu Leu Ile Pro Leu Val Arg
115 120

<210> 31
<211> 278
<212> DNA
<213> Zea sp

<400> 31
tattcagcac cacctctcaa gctcaagcag aatggatgga ttgggaactt cgctctgggt 60
gcgagttaca tcagcttgcc ctggtgggct ggccaggcgt tatttggaac tcttacacca 120
gatatcattg tcttgactac tttgtacagc atagctgggc tagggattgc tattgtaaat 180
gatttcaaga gtattgaagg ggataggact ctggggcttc agtcacttcc tgttgctttt 240
gggatggaaa ctgcaaaatg gatttgtgtt ggagcaat 278

<210> 32
<211> 292
<212> PRT
<213> Synechocystis sp

<400> 32
Met Val Ala Gln Thr Pro Ser Ser Pro Pro Leu Trp Leu Thr Ile Ile
1 5 10 15
Tyr Leu Leu Arg Trp His Lys Pro Ala Gly Arg Leu Ile Leu Met Ile
20 25 30
Pro Ala Leu Trp Ala Val Cys Leu Ala Ala Gln Gly Leu Pro Pro Leu
35 40 45
Pro Leu Leu Gly Thr Ile Ala Leu Gly Thr Leu Ala Thr Ser Gly Leu
50 55 60
Gly Cys Val Val Asn Asp Leu Trp Asp Arg Asp Ile Asp Pro Gln Val
65 70 75 80
Glu Arg Thr Lys Gln Arg Pro Leu Ala Ala Arg Ala Leu Ser Val Gln
85 90 95
Val Gly Ile Gly Val Ala Leu Val Ala Leu Leu Cys Ala Ala Gly Leu
100 105 110
Ala Phe Tyr Leu Thr Pro Leu Ser Phe Trp Leu Cys Val Ala Ala Val
115 120 125
Pro Val Ile Val Ala Tyr Pro Gly Ala Lys Arg Val Phe Pro Val Pro
130 135 140

Gln Leu Val Leu Ser Ile Ala Trp Gly Phe Ala Val Leu Ile Ser Trp
145 150 155 160
Ser Ala Val Thr Gly Asp Leu Thr Asp Ala Thr Trp Val Leu Trp Gly
165 170 175
Ala Thr Val Phe Trp Thr Leu Gly Phe Asp Thr Val Tyr Ala Met Ala
180 185 190
Asp Arg Glu Asp Asp Arg Arg Ile Gly Val Asn Ser Ser Ala Leu Phe
195 200 205
Phe Gly Gln Tyr Val Gly Glu Ala Val Gly Ile Phe Phe Ala Leu Thr
210 215 220
Ile Gly Cys Leu Phe Tyr Leu Gly Met Ile Leu Met Leu Asn Pro Leu
225 230 235 240
Tyr Trp Leu Ser Leu Ala Ile Ala Ile Val Gly Trp Val Ile Gln Tyr
245 250 255
Ile Gln Leu Ser Ala Pro Thr Pro Glu Pro Lys Leu Tyr Gly Gln Ile
260 265 270
Phe Gly Gln Asn Val Ile Ile Gly Phe Val Leu Leu Ala Gly Met Leu
275 280 285
Leu Gly Trp Leu
290

<210> 33
<211> 316
<212> PRT
<213> Synechocystis sp

<400> 33
Met Val Thr Ser Thr Lys Ile His Arg Gln His Asp Ser Met Gly Ala
1 5 10 15
Val Cys Lys Ser Tyr Tyr Gln Leu Thr Lys Pro Arg Ile Ile Pro Leu
20 25 30
Leu Leu Ile Thr Thr Ala Ala Ser Met Trp Ile Ala Ser Glu Gly Arg
35 40 45
Val Asp Leu Pro Lys Leu Leu Ile Thr Leu Leu Gly Gly Thr Leu Ala
50 55 60
Ala Ala Ser Ala Gln Thr Leu Asn Cys Ile Tyr Asp Gln Asp Ile Asp
65 70 75 80
Tyr Glu Met Leu Arg Thr Arg Ala Arg Pro Ile Pro Ala Gly Lys Val
85 90 95
Gln Pro Arg His Ala Leu Ile Phe Ala Leu Ala Leu Gly Val Leu Ser
100 105 110
Phe Ala Leu Leu Ala Thr Phe Val Asn Val Leu Ser Gly Cys Leu Ala
115 120 125
Leu Ser Gly Ile Val Phe Tyr Met Leu Val Tyr Thr His Trp Leu Lys
130 135 140
Arg His Thr Ala Gln Asn Ile Val Ile Gly Gly Ala Ala Gly Ser Ile

145 150 155 160
Pro Pro Leu Val Gly Trp Ala Ala Val Thr Gly Asp Leu Ser Trp Thr
165 170 175
Pro Trp Val Leu Phe Ala Leu Ile Phe Leu Trp Thr Pro Pro His Phe
180 185 190
Trp Ala Leu Ala Leu Met Ile Lys Asp Asp Tyr Ala Gln Val Asn Val
195 200 205
Pro Met Leu Pro Val Ile Ala Gly Glu Glu Lys Thr Val Ser Gln Ile
210 215 220
Trp Tyr Tyr Ser Leu Leu Val Val Pro Phe Ser Leu Leu Leu Val Tyr
225 230 235 240
Pro Leu His Gln Leu Gly Ile Leu Tyr Leu Ala Ile Ala Ile Ile Leu
245 250 255
Gly Gly Gln Phe Leu Val Lys Ala Trp Gln Leu Lys Gln Ala Pro Gly
260 265 270
Asp Arg Asp Leu Ala Arg Gly Leu Phe Lys Phe Ser Ile Phe Tyr Leu
275 280 285
Met Leu Leu Cys Leu Ala Met Val Ile Asp Ser Leu Pro Val Thr His
290 295 300
Gln Leu Val Ala Gln Met Gly Thr Leu Leu Leu Gly
305 310 315

<210> 34
<211> 324
<212> PRT
<213> Synechocystis sp

<400> 34
Met Ser Asp Thr Gln Asn Thr Gly Gln Asn Gln Ala Lys Ala Arg Gln
1 5 10 15
Leu Leu Gly Met Lys Gly Ala Ala Pro Gly Glu Ser Ser Ile Trp Lys
20 25 30
Ile Arg Leu Gln Leu Met Lys Pro Ile Thr Trp Ile Pro Leu Ile Trp
35 40 45
Gly Val Val Cys Gly Ala Ala Ser Ser Gly Gly Tyr Ile Trp Ser Val
50 55 60
Glu Asp Phe Leu Lys Ala Leu Thr Cys Met Leu Leu Ser Gly Pro Leu
65 70 75 80
Met Thr Gly Tyr Thr Gln Thr Leu Asn Asp Phe Tyr Asp Arg Asp Ile
85 90 95
Asp Ala Ile Asn Glu Pro Tyr Arg Pro Ile Pro Ser Gly Ala Ile Ser
100 105 110
Val Pro Gln Val Val Thr Gln Ile Leu Ile Leu Leu Val Ala Gly Ile
115 120 125
Gly Val Ala Tyr Gly Leu Asp Val Trp Ala Gln His Asp Phe Pro Ile
130 135 140

Met Met Val Leu Thr Leu Gly Gly Ala Phe Val Ala Tyr Ile Tyr Ser
145 150 155 160
Ala Pro Pro Leu Lys Leu Lys Gln Asn Gly Trp Leu Gly Asn Tyr Ala
165 170 175
Leu Gly Ala Ser Tyr Ile Ala Leu Pro Trp Trp Ala Gly His Ala Leu
180 185 190
Phe Gly Thr Leu Asn Pro Thr Ile Met Val Leu Thr Leu Ile Tyr Ser
195 200 205
Leu Ala Gly Leu Gly Ile Ala Val Val Asn Asp Phe Lys Ser Val Glu
210 215 220
Gly Asp Arg Gln Leu Gly Leu Lys Ser Leu Pro Val Met Phe Gly Ile
225 230 235 240
Gly Thr Ala Ala Trp Ile Cys Val Ile Met Ile Asp Val Phe Gln Ala
245 250 255
Gly Ile Ala Gly Tyr Leu Ile Tyr Val His Gln Gln Leu Tyr Ala Thr
260 265 270
Ile Val Leu Leu Leu Leu Ile Pro Gln Ile Thr Phe Gln Asp Met Tyr
275 280 285
Phe Leu Arg Asn Pro Leu Glu Asn Asp Val Lys Tyr Gln Ala Ser Ala
290 295 300
Gln Pro Phe Leu Val Phe Gly Met Leu Ala Thr Gly Leu Ala Leu Gly
305 310 315 320
His Ala Gly Ile

<210> 35
<211> 307
<212> PRT
<213> Synechocystis sp

<400> 35
Met Thr Glu Ser Ser Pro Leu Ala Pro Ser Thr Ala Pro Ala Thr Arg
1 5 10 15
Lys Leu Trp Leu Ala Ala Ile Lys Pro Pro Met Tyr Thr Val Ala Val
20 25 30
Val Pro Ile Thr Val Gly Ser Ala Val Ala Tyr Gly Leu Thr Gly Gln
35 40 45
Trp His Gly Asp Val Phe Thr Ile Phe Leu Leu Ser Ala Ile Ala Ile
50 55 60
Ile Ala Trp Ile Asn Leu Ser Asn Asp Val Phe Asp Ser Asp Thr Gly
65 70 75 80
Ile Asp Val Arg Lys Ala His Ser Val Val Asn Leu Thr Gly Asn Arg
85 90 95
Asn Leu Val Phe Leu Ile Ser Asn Phe Phe Leu Leu Ala Gly Val Leu
100 105 110
Gly Leu Met Ser Met Ser Trp Arg Ala Gln Asp Trp Thr Val Leu Glu

115 120 125

Leu Ile Gly Val Ala Ile Phe Leu Gly Tyr Thr Tyr Gln Gly Pro Pro
130 135 140

Phe Arg Leu Gly Tyr Leu Gly Leu Gly Glu Leu Ile Cys Leu Ile Thr
145 150 155 160

Phe Gly Pro Leu Ala Ile Ala Ala Ala Tyr Tyr Ser Gln Ser Gln Ser
165 170 175

Phe Ser Trp Asn Leu Leu Thr Pro Ser Val Phe Val Gly Ile Ser Thr
180 185 190

Ala Ile Ile Leu Phe Cys Ser His Phe His Gln Val Glu Asp Asp Leu
195 200 205

Ala Ala Gly Lys Lys Ser Pro Ile Val Arg Leu Gly Thr Lys Leu Gly
210 215 220

Ser Gln Val Leu Thr Leu Ser Val Val Ser Leu Tyr Leu Ile Thr Ala
225 230 235 240

Ile Gly Val Leu Cys His Gln Ala Pro Trp Gln Thr Leu Leu Ile Ile
245 250 255

Ala Ser Leu Pro Trp Ala Val Gln Leu Ile Arg His Val Gly Gln Tyr
260 265 270

His Asp Gln Pro Glu Gln Val Ser Asn Cys Lys Phe Ile Ala Val Asn
275 280 285

Leu His Phe Phe Ser Gly Met Leu Met Ala Ala Gly Tyr Gly Trp Ala
290 295 300

Gly Leu Gly
305

<210> 36
<211> 927
<212> DNA
<213> Synechocystis sp

<400> 36
atggcaacta tccaagcttt ttggcgcttc tcccgccccc ataccatcat tggtacaact 60
ctgagcgtct gggctgtgta tctgttaact attctcgggg atggaaactc agttaactcc 120
cctgcttccc tggatttagt gttcggcgct tggctggcct gcctgttggg taatgtgtac 180
attgtcggcc tcaaccaatt gtgggatgtg gacattgacc gcatcaataa gccgaatttg 240
cccctagcta acggagattt ttctatcgcc cagggccgtt ggattgtggg actttgtggc 300
gttgcttcct tggcgatcgc ctggggatta gggctatggc tggggctaac ggtgggcatt 360
agtttgatta ttggcacggc ctattcggtg ccgccagtga ggttaaagcg cttttccctg 420
ctggcggccc tgtgtattct gacggtgcgg ggaattgtgg ttaacttggg cttatttta 480
ttttttagaa ttggtttagg ttatcccccc actttaataa cccccatctg ggttttgact 540
ttatttatct tagttttcac cgtggcgatc gccatttta aagatgtgcc agatatggaa 600
ggcgatcggc aatttaagat tcaaacttta actttgcaaa tcggcaaaca aaacgttttt 660
cggggaacct taattttact cactggttgt tatttagcca tggcaatctg gggcttatgg 720
gcggctatgc ctttaaatac tgctttcttg attgtttccc atttgtgctt attagcctta 780
ctctggtggc ggagtcgaga tgtacactta gaaagcaaaa ccgaaattgc tagtttttat 840

cagtttattt ggaagctatt tttcttagag tacttgctgt atcccttggc tctgtggtta 900
cctaattttt ctaatactat tttttag 927

<210> 37
<211> 308
<212> PRT
<213> Synechocystis sp

<400> 37

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Met Ala Thr Ile Gln Ala Phe Trp Arg Phe Ser Arg Pro His Thr Ile
 1               5                  10                  15
Ile Gly Thr Thr Leu Ser Val Trp Ala Val Tyr Leu Leu Thr Ile Leu
            20                  25                  30
Gly Asp Gly Asn Ser Val Asn Ser Pro Ala Ser Leu Asp Leu Val Phe
        35                  40                  45
Gly Ala Trp Leu Ala Cys Leu Leu Gly Asn Val Tyr Ile Val Gly Leu
    50                  55                  60
Asn Gln Leu Trp Asp Val Asp Ile Asp Arg Ile Asn Lys Pro Asn Leu
65                  70                  75                  80
Pro Leu Ala Asn Gly Asp Phe Ser Ile Ala Gln Gly Arg Trp Ile Val
                85                  90                  95
Gly Leu Cys Gly Val Ala Ser Leu Ala Ile Ala Trp Gly Leu Gly Leu
            100                 105                 110
Trp Leu Gly Leu Thr Val Gly Ile Ser Leu Ile Ile Gly Thr Ala Tyr
        115                 120                 125
Ser Val Pro Pro Val Arg Leu Lys Arg Phe Ser Leu Leu Ala Ala Leu
    130                 135                 140
Cys Ile Leu Thr Val Arg Gly Ile Val Val Asn Leu Gly Leu Phe Leu
145                 150                 155                 160
Phe Phe Arg Ile Gly Leu Gly Tyr Pro Pro Thr Leu Ile Thr Pro Ile
                165                 170                 175
Trp Val Leu Thr Leu Phe Ile Leu Val Phe Thr Val Ala Ile Ala Ile
            180                 185                 190
Phe Lys Asp Val Pro Asp Met Glu Gly Asp Arg Gln Phe Lys Ile Gln
        195                 200                 205
Thr Leu Thr Leu Gln Ile Gly Lys Gln Asn Val Phe Arg Gly Thr Leu
    210                 215                 220
Ile Leu Leu Thr Gly Cys Tyr Leu Ala Met Ala Ile Trp Gly Leu Trp
225                 230                 235                 240
Ala Ala Met Pro Leu Asn Thr Ala Phe Leu Ile Val Ser His Leu Cys
                245                 250                 255
Leu Leu Ala Leu Leu Trp Trp Arg Ser Arg Asp Val His Leu Glu Ser
            260                 265                 270
Lys Thr Glu Ile Ala Ser Phe Tyr Gln Phe Ile Trp Lys Leu Phe Phe
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290 295 300

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Ser Phe Ala Phe Met Tyr Ser Ile Glu Asn Pro Ala Ser Asp His His
35 40 45
Tyr Gly Gly Gly Ala Val Gln Ile Leu Gly Pro Ala Thr Lys Lys Gln
50 55 60
Glu Asn Gln Glu Asp Gln Leu Val Trp Arg Thr Phe Pro Ser Val Lys
65 70 75 80

Lys Phe Trp Ala Ser Pro Arg Gln Phe Ala Leu Gly His Trp Gly Lys
85 90 95
Cys Arg Asp Asn Arg Gln Ala Lys Pro Leu Leu Ser Glu Glu Phe Phe
100 105 110
Ala Thr Val Lys Glu Gly Tyr Gln Ile His Gln Asn Gln His Gln Gly
115 120 125
Gln Ile Ile His Gly Asp Arg His Cys Arg Trp Gln Phe Thr Val Glu
130 135 140
Pro Glu Val Thr Trp Gly Ser Pro Asn Arg Phe Pro Arg Ala Thr Ala
145 150 155 160
Gly Trp Leu Ser Phe Leu Pro Leu Phe Asp Pro Gly Trp Gln Ile Leu
165 170 175
Leu Ala Gln Gly Arg Ala His Gly Trp Leu Lys Trp Gln Arg Glu Gln
180 185 190
Tyr Glu Phe Asp His Ala Leu Val Tyr Ala Glu Lys Asn Trp Gly His
195 200 205
Ser Phe Pro Ser Arg Trp Phe Trp Leu Gln Ala Asn Tyr Phe Pro Asp
210 215 220
His Pro Gly Leu Ser Val Thr Ala Ala Gly Gly Glu Arg Ile Val Leu
225 230 235 240
Gly Arg Pro Glu Glu Val Ala Leu Ile Gly Leu His His Gln Gly Asn
245 250 255
Phe Tyr Glu Phe Gly Pro Gly His Gly Thr Val Thr Trp Gln Val Ala
260 265 270
Pro Trp Gly Arg Trp Gln Leu Lys Ala Ser Asn Asp Arg Tyr Trp Val
275 280 285
Lys Leu Ser Gly Lys Thr Asp Lys Lys Gly Ser Leu Val His Thr Pro
290 295 300
Thr Ala Gln Gly Leu Gln Leu Asn Cys Arg Asp Thr Thr Arg Gly Tyr
305 310 315 320
Leu Tyr Leu Gln Leu Gly Ser Val Gly His Gly Leu Ile Val Gln Gly
325 330 335
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<210> 43
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<210> 44
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<400> 44
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<400> 45
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<210> 46
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<400> 47
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<210> 90
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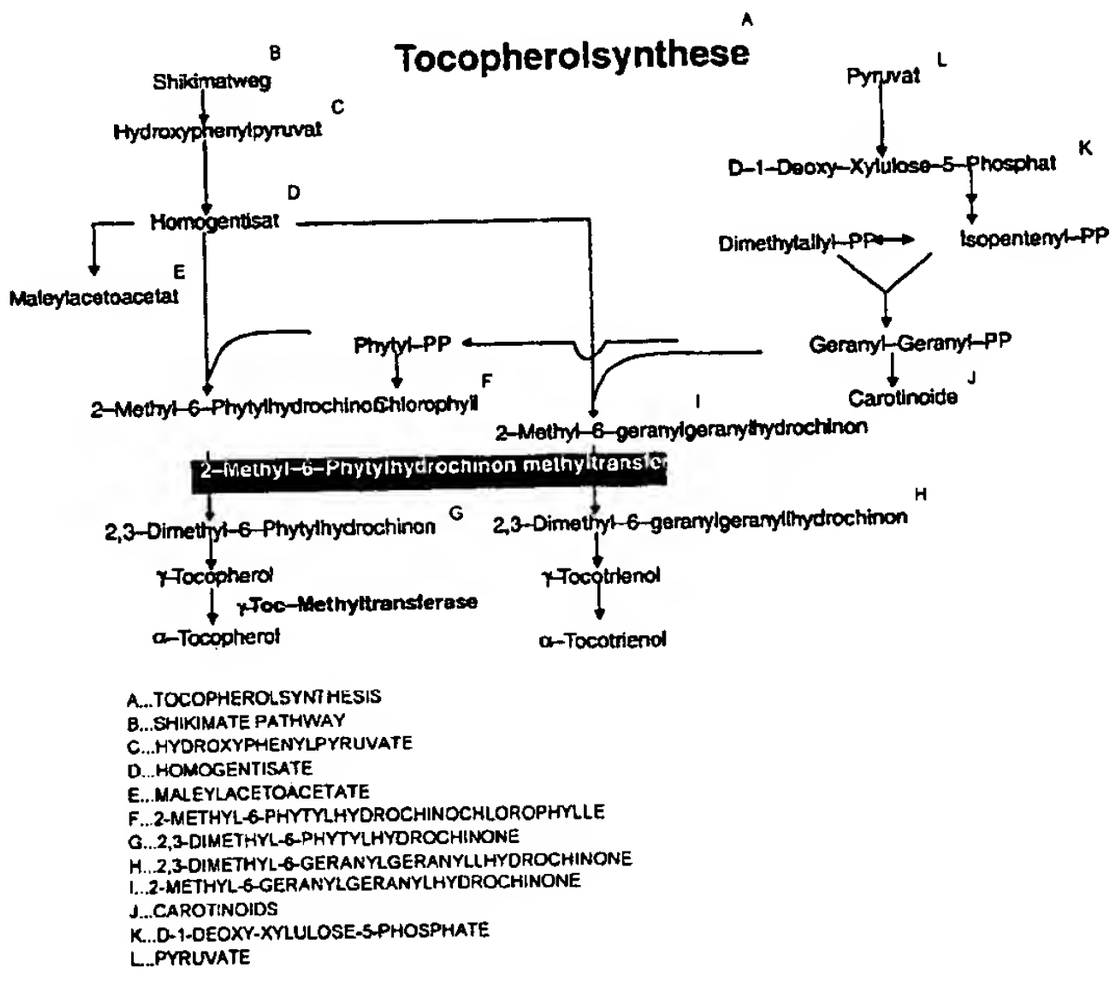
(81) Bestimmungsstaaten *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

[Fortsetzung auf der nächsten Seite]

(54) Title: IDENTIFICATION AND OVEREXPRESSION OF A DNA SEQUENCE CODING FOR 2-METHYL-6-PHYTYLHYDROQUINONE-METHYLTRANSFERASE IN PLANTS

(54) Bezeichnung: IDENTIFIZIERUNG UND ÜBEREXPRESSION EINER DNA-SEQUENZ KODIEREND FÜR EINE 2-METHYL-6-PHYTYLHYDROCHINON-METHYLTRANSFERASE IN PFLANZEN



(57) **Abstract:** A method for the prdouctin of plants with an increased tocipherol and tocotrienol content by overexpression of a gene coding for 2-methyl-6-phytylhydroquinone-methyltransferase.

(57) **Zusammenfassung:** Verfahren zur Herstellung von Pflanzen mit erhöhtem Gehalt an Tocopherolen und Tocotrienolen durch Überexpression eines Gens codierend für eine 2-Mehtyl-6-phytylhydrochinon-methyltransferase.

WO 01/04330 A1

REF A44

Della Penna, et al.
S/N 09/560,761

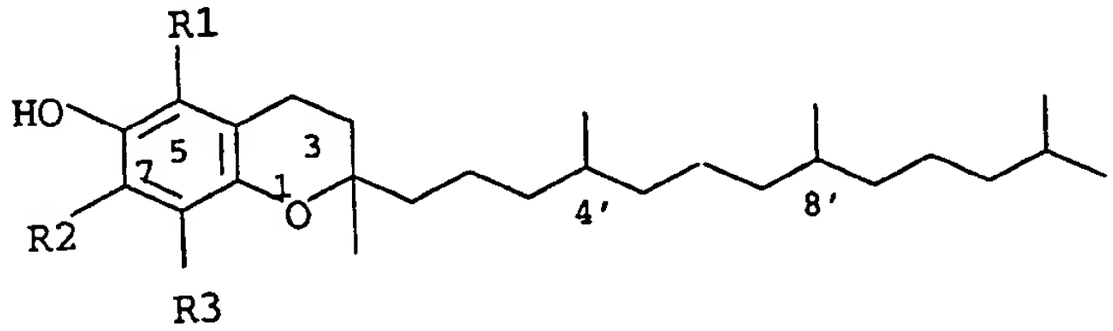
Identifizierung und Überexpression einer DNA-Sequenz codierend für eine 2-Methyl-6-phytylhydrochinon-methyltransferase in Pflanzen

Beschreibung

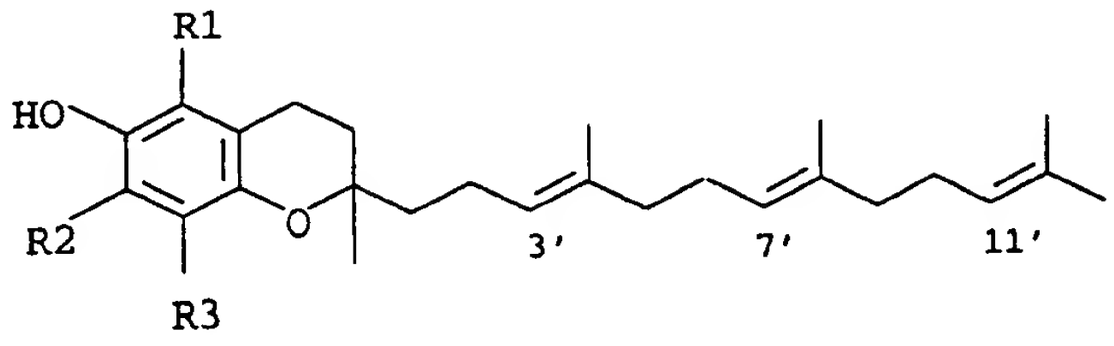
Die Erfindung betrifft eine DNA kodierend für ein Polypeptid mit 2-Methyl-6-phytylhydrochinon-methyltransferase Aktivität. Zudem betrifft die Erfindung die Verwendung von DNA-Sequenzen codierend für ein Polypeptid mit 2-Methyl-6-phytylhydrochinon-Methyltransferase Aktivität zur Herstellung von Pflanzen mit erhöhtem Gehalt an Tocopherolen und Tocotrienolen, speziell die Verwendung der DNA-Sequenz SEQ-ID Nr. 1 oder SEQ-ID Nr. 7 oder mit dieser hybridisierende oder zur Gesamtsequenz oder zu Teilsequenzen homologen DNA-Sequenzen, einem Verfahren zur Herstellung von Pflanzen mit erhöhtem Gehalt an Tocopherolen und Tocotrienolen, sowie die derart hergestellte Pflanze selbst.

Ein wichtiges Ziel pflanzenmolekulargenetischer Arbeiten ist bisher die Erzeugung von Pflanzen mit erhöhtem Gehalt an Zuckern, Enzymen und Aminosäuren. Wirtschaftlich interessant ist jedoch auch die Entwicklung von Pflanzen mit erhöhtem Gehalt an Vitaminen, wie z.B. der Erhöhung des Tocopherol- und Tocotrienolgehaltes.

Die in der Natur vorkommenden acht Verbindungen mit Vitamin E-Aktivität sind Derivate des 6-Chromanols (Ullmann's Encyclopedia of Industrial Chemistry, Vol. A 27 (1996), VCH Verlagsgesellschaft, Chapter 4., 478-488, Vitamin E). Die erste Gruppe (1a-d) stammt von Tocopherol ab, die zweite Gruppe besteht aus Derivaten des Tocotrienols (2a- d):



1a, α-Tocopherol: $R^1 = R^2 = R^3 = CH_3$
1b, β-Tocopherol [148-03-8]: $R^1 = R^3 = CH_3$, $R^2 = H$
1c, γ-Tocopherol [54-28-4]: $R^1 = H$, $R^2 = R^3 = CH_3$
1d, δ-Tocopherol [119-13-1]: $R^1 = R^2 = H$, $R^3 = CH_3$



2a, α-Tocotrienol [1721-51-3]: $R^1 = R^2 = R^3 = CH_3$
2b, β-Tocotrienol [490-23-3]: $R^1 = R^3 = CH_3$, $R^2 = H$
2c, γ-Tocotrienol [14101-61-2]: $R^1 = H$, $R^2 = R^3 = CH_3$
2d, δ-Tocotrienol [25612-59-3]: $R^1 = R^2 = H$, $R^3 = CH_3$

Wirtschaftlich große Bedeutung besitzt α-Tocopherol.

Der Entwicklung von Kulturpflanzen mit erhöhtem Gehalt an Tocopherolen und Tocotrienolen durch klassische Züchtungsmethoden sind Grenzen gesetzt.

Eine sinnvolle Alternative ist das gentechnische Vorgehen, beispielsweise die für die Tocopherol Syntheseleistung kodierenden, essentiellen Biosynthesegene zu isolieren und in Kulturpflanzen gezielt zu übertragen. Dieses Verfahren setzt voraus, daß die Biosynthese und deren Regulation bekannt ist und daß Gene, die die Biosyntheseleistung beeinflussen, identifiziert werden.

Isoprenoide oder Terpenoide bestehen aus verschiedenen Klassen lipidlöslicher Moleküle und werden teilweise oder vollständig aus C_5-Isopren-Einheiten gebildet. Reine Prenyllipide (z.B. Carotinoide) bestehen aus C-Gerüsten, die ausschließlich auf Isopren-Einheiten zurückgehen, während gemischte Prenyllipide (z.B. Chlorophylle, Tocopherole und Vitamin K) eine Isoprenoid-Seitenkette besitzen, die mit einem aromatischen Kern verbunden ist.

Ausgangspunkt der Biosynthese von Prenyllipiden sind 3 x Acetyl-CoA Einheiten, die über ß-Hydroxymethylglutaryl-CoA (HMG-CoA) und Mevalonat in die Ausgangs-Isopren-Einheit (C5), dem Isopentenylpyrophosphat (IPP), umgewandelt werden. Kürzlich wurde durch *in vivo* Fütterungsexperimente mit C^{13} gezeigt, daß in verschiedenen Eubakterien, Grünalgen und pflanzlichen Chloroplasten ein Mevalonat-unabhängiger Weg zur Bildung von IPP beschritten wird. Dabei werden Hydroxyethylthiamin, das durch Decarboxylierung von Pyruvat entsteht, und Glycerinaldehyd-3-Phosphat (3-GAP) in einer durch die 1-Deoxy-D-Xylulose-5-Phosphat Synthase vermittelten "Transketolase"-Reaktion zunächst in 1-Deoxy-D-Xylulose-5-phosphat umgewandelt (Lange et al, 1998; Schwender et al, 1997; Arigoni et al, 1997; Lichtenthaler et al, 1997; Sprenger et

al, 1997). Dieses wird dann durch eine intramolekulare Umordnung in 2-C-Methyl-D-Erythritol-4-Phosphat und im weiteren zu IPP umgesetzt (Arigoni et al, 1997; Zeidler et al, 1998). Biochemische Daten deuten darauf hin, daß der Mevalonat-Weg im Zytosol operiert und zur Bildung von Phytosterolen führt. Das Antibiotikum Mevinolin, ein spezifischer Inhibitor der Mevalonat-Bildung, führt lediglich zur Inhibition der Sterol-Biosynthese im Zytoplasma, während die Prenyllipid-Bildung in den Plastiden unbeeinflußt ist (Bach & Lichtenthaler, 1993). Der Mevalonat-unabhängige Weg ist dagegen plastidär lokalisiert und führt vornehmlich zur Bildung von Carotinoiden und plastidären Prenyllipiden (Schwender et al, 1997; Arigoni et al, 1997).

IPP steht im Gleichgewicht mit seinem Isomer, dem Dimethylallyl Pyrophosphat (DMAPP). Eine Kondensation von IPP mit DMAPP in Kopf-Schwanz Anlagerung ergibt das Monoterpen (C10) Geranyl-Pyrophosphat (GPP). Die Addition von weiteren IPP Einheiten führt zum Sesquiterpen (C15) Farnesy-Pyrophosphat (FPP) und zum Diterpen (C20) Geranyl-Geranyl-Pyrophosphat (GGPP). Die Verknüpfung zweier GGPP Moleküle führt zur Bildung der C40-Vorläufer für Carotinoide.

Bei gemischten Prenyllipiden ist die Isopren-Seitenkette verschiedener Länge mit Nicht-Isopren Ringen verbunden wie beispielsweise ein Porphyrin-Ring bei Chlorophyll a und b. Die Chlorophylle und Phylloquinone enthalten eine C20 Phytyl-Kette, in der nur die erste Isopren-Einheit eine Doppelbindung enthält. GGPP wird durch die Geranylgeranyl-Pyrophosphat-Oxidoreduktase (GGPPOR) zum Phytyl-Pyrophosphat (PPP) umgeformt, dem Ausgangsstoff für die weitere Bildung von Tocopherolen.

Bei den Ringstrukturen der gemischten Prenyllipide, die zur Bildung der Vitamine E und K führen, handelt es sich um Quinone, deren Ausgangsmetabolite aus dem Shikimat-Weg stammen. Die aromatischen Aminosäuren Phenylalanin bzw. Tyrosin werden in Hydroxyphenyl-Pyruvat umgewandelt, welches durch Dioxygenierung in Homogentisinsäure überführt wird. Das Chorismat wird ausgehend von Erythrose-4-Phosphat und Phosphoenolpyruvat (PEP) durch deren Kondensation zu 3-deoxy-D-Arabino-heptulosonat-7-Phosphat (DAHP) über die Zwischenstufen des Shikimatweges 3'-Dehydroquinat, 3'-Dehydroshikimat, Shikimat, Shikimat-3-Phosphat und 5'-Enolpyruvylshikimat-3-Phosphat gebildet. Dabei wird das Erythrose-4-Phosphat vom Calvinzyklus gebildet und das PEP von der Glykolyse bereitgestellt. Die oben beschriebene Homogentisinsäure wird anschließend an Phytylpyrophosphat (PPP) bzw. Geranylgeranylpyrophosphat gebunden, um die Vorläufer von α-Tocopherol und α-Tocotrienol, das 2-Methyl-6-phytylhydrochinon bzw. das

2-Methyl-6-geranylgeranylhydrochinon zu bilden. Durch Methylierungsschritte mit S-Adenosylmethionin als Methyl-Gruppen-Donor entsteht zunächst 2,3-Dimethyl-6-phytylquinol, dann durch Zyklisierung α-Tocopherol und durch nochmalige Methylierung α-Tocopherol (Richter, Biochemie der Pflanzen, Georg Thieme Verlag Stuttgart, 1996).

In der Literatur finden sich Beispiele die zeigen, daß die Manipulation eines Enzyms den Metabolit-Fluß direktional beeinflußen kann. In Experimenten mit einer veränderten Expression der Phytoen Synthase, welche zwei GGPP-Moleküle zu 15-cis-Phytoen miteinander verknüpft, konnte ein direkter Einfluß auf die Carotinoid-Mengen dieser transgenen Tomatenpflanzen gemessen werden (Fray und Grierson, Plant Mol.Biol.22(4),589-602(1993); Fray et al., Plant J., 8, 693-701(1995)). Wie zu erwarten, zeigen transgene Tabakpflanzen mit verringerten Mengen an Phenylalanin-Ammonium Lyase reduzierte Phenylpropanoid-Mengen. Das Enzym Phenylalanin-Ammonium Lyase katalysiert den Abbau von Phenylalanin, entzieht es also der Phenylpropanoid-Biosynthese (Bate et al.,Proc. Natl. Acad. Sci USA 91 (16): 7608-7612 (1994); Howles et al., Plant Physiol. 112. 1617-1624(1996)).

Über die Erhöhung des Metabolitflusses zur Steigerung des Tocopherol- bzw. Tocotrienolgehaltes in Pflanzen durch Überexpression einzelner Biosynthesegene ist bisher wenig bekannt. Lediglich WO 97/27285 beschreibt eine Modifikation des Tocopherol-Gehaltes durch verstärkte Expression bzw. durch Herunterregulation des Enzyms p-Hydroxyphenylpyruvatdioxygenase (HPPD). WO 99/04622 beschreibt eine Gensequenz codierend für eine γ-Tocopherolmethyltransferase aus einem photosynthetisch aktiven Organismus. WO 99/23231 zeigt, daß die Expression einer Geranylgeranyl-Reductase in transgenen Pflanzen eine gesteigerte Tocopherolbiosynthese zur Folge hat.

Aufgabe der vorliegenden Erfindung war die Entwicklung einer transgenen Pflanze mit erhöhtem Gehalt an Tocopherolen und Tocotrienolen.

Die Aufgabe wurden überraschenderweise gelöst durch die Überexpression eines 2-Methyl-6-phytylhydrochinon-methyltransferase Gens in Pflanzen.

Zu diesem Zweck wurde in transgenen Pflanzen die Aktivität der 2-Methyl-6-phytylhydrochinon-methyltransferase (MPMT) durch Überexpression des MPMT-Gens aus Synechocystis spec. PCC 6803 er-

höht. Dies kann prinzipiell durch Expression homologer oder heterologer MPMT-Gene erreicht werden.

In Beispiel 2 wird erstmals die Klonierung einer MPMT-DNA-Sequenz (SEQ-ID Nr. 1) aus Synechocystis spec. PCC 6803 beschrieben. Um eine Plastidenlokalisation zu gewährleisten wird der MPMT-Nukleotidsequenz aus Synechocystis eine Transitsignalsequenz (Abb. 3, Abb. 4) vorangestellt. Auch geeignet als Expressionskassette ist eine DNA-Sequenz, die für ein MPMT-Gen codiert, das mit SEQ-ID Nr. 1 hybridisiert, bzw. zur Gesamtsequenz oder zu Teilsequenzen homolog ist und das aus anderen Organismen bzw. aus Pflanzen stammt.

Das durch die zusätzliche Expression des MPMT-Gens nun vermehrt zur Verfügung stehende 2,3-Dimethyl-6-phytylhydrochinon wird weiter in Richtung Tocopherole und Tocotrienol umgesetzt (Abbildung 1).

Die Herstellung der transgenen Pflanzen erfolgt durch Transformation der Pflanzen mit einem das MPMT-Gen enthaltenden Konstrukt. Als Modellpflanzen für die Produktion von Tocopherolen und Tocotrienolen wurden Arabidopsis thaliana, Brassica napus und Nicotiana tabacum eingesetzt.

Messungen an MPMT-Synechocystis knock out Mutanten ergaben bezüglich des Gehaltes an Tocopherolen und Tocotrienolen eine drastische Abnahme. Dies belegt den direkten Einfluß der plastidären pflanzlichen MPMT auf die Synthese von Tocopherolen und Tocotrienolen.

Gegenstand der Erfindung ist die Verwendung einer DNA-Sequenz SEQ-ID Nr. 1 aus Synechocystis spec. PCC 6803, die für eine MPMT oder deren funktionelle Äquivalente kodiert, zur Herstellung einer Pflanze mit erhöhtem Gehalt an Tocopherolen und Tocotrienolen. Die Nukleinsäuresequenz kann dabei z.B. eine DNA- oder cDNA-Sequenz sein. Zur Insertion in eine Expressionskassette geeignete kodierende Sequenzen sind beispielsweise solche, die für eine MPMT kodieren und die dem Wirt die Fähigkeit zur Überproduktion von Tocopherolen und Tocotrienolen verleihen.

Die Expressionskassetten beinhalten außerdem regulative Nukleinsäuresequenzen, welche die Expression der kodierenden Sequenz in der Wirtszelle steuern. Gemäß einer bevorzugten Ausführungsform umfaßt eine Expressionskassette stromaufwärts, d.h. am 5'-Ende der kodierenden Sequenz, einen Promotor und stromabwärts, d.h. am 3'-Ende, ein Polyadenylierungssignal und gegebenenfalls weitere regulatorische Elemente, welche mit der dazwischenliegenden ko-

dierenden Sequenz für das MPMT-Gen operativ verknüpft sind. Unter einer operativen Verknüpfung versteht man die sequenzielle Anordnung von Promotor, kodierender Sequenz, Terminator und ggf. weiterer regulativer Elemente derart, daß jedes der regulativen Elemente seine Funktion bei der Expression der kodierenden Sequenz bestimmungsgemäß erfüllen kann. Die zur operativen Verknüpfung bevorzugten aber nicht darauf beschränkten Sequenzen sind Targeting-Sequenzen zur Gewährleistung der subzellulären Lokalisation im Apoplasten, in der Vakuole, in Plastiden, im Mitochondrium, im Endoplasmatischen Retikulum (ER), im Zellkern, in Ölkörperchen oder anderen Kompartimenten und Translationsverstärker wie die 5'-Führungssequenz aus dem Tabak-Mosaik-Virus (Gallie et al., Nucl. Acids Res. 15 (1987), 8693 -8711).

Beispielhaft kann die pflanzliche Expressionskassette in ein Derivat des Transformationsvektors pBin-19 mit 35s Promotor (Bevan, M., Nucleic Acids Research 12: 8711-8721 (1984)) eingebaut werden. Abbildung 4 zeigt ein Derivat des Transformationsvektors pBin -19 mit samenspezifischem Legumin B4-Promotor.

Als Promotoren der Expressionskassette ist grundsätzlich jeder Promotor geeignet, der die Expression von Fremdgenen in Pflanzen steuern kann. Vorzugsweise verwendet man insbesondere einen pflanzlichen Promotor oder einen Promotor, der einem Pflanzenvirus entstammt. Insbesondere bevorzugt ist der CaMV 35S-Promotor aus dem Blumenkohl-Mosaik-Virus (Franck et al., Cell 21 (1980), 285 - 294). Dieser Promotor enthält bekanntlich unterschiedliche Erkennungssequenzen für transkriptionale Effektoren, die in ihrer Gesamtheit zu einer permanenten und konstitutiven Expression des eingeführten Gens führen (Benfey et al., EMBO J. 8 (1989), 2195-2202).

Die Expressionskassette kann auch einen chemisch induzierbaren Promotor enthalten, durch den die Expression des exogenen MPMT-Gens in der Pflanze zu einem bestimmten Zeitpunkt gesteuert werden kann. Derartige Promotoren wie z.B. der PRP1-Promotor (Ward et al., Plant. Mol. Biol. 22 (1993), 361-366), ein durch Salizylsäure induzierbarer Promotor (WO 95/19443), ein durch Benzenesulfonamid-induzierbarer (EP-A 388186), ein durch Tetrazyklin-induzierbarer (Gatz et al., (1992) Plant J. 2, 397-404), ein durch Abscisinsäure-induzierbarer (EP-A 335528) bzw. ein durch Ethanol- oder Cyclohexanon-induzierbarer (WO 93/21334) Promotor können u.a. verwendet werden.

Weiterhin sind insbesonders solche Promotoren bevorzugt, die die Expression in Geweben oder Pflanzenteilen sicherstellen, in denen beispielsweise die Biosynthese von Tocopherol bzw. dessen Vorstu-

fen stattfindet. Insbesondere zu nennen sind Promotoren, die eine blattspezifische Expression gewährleisten. Zu nennen sind der Promotor der cytosolischen FBPase aus Kartoffel oder der ST-LSI Promotor aus Kartoffel (Stockhaus et al., EMBO J. 8 (1989), 2445 - 245).

Mit Hilfe eines samenspezifischen Promotors konnte ein Fremdprotein stabil bis zu einem Anteil von 0,67 % des gesamten löslichen Samenproteins in den Samen transgener Tabakpflanzen exprimiert werden (Fiedler und Conrad, Bio/Technology 10 (1995), 1090-1094). Die Expressionskassette kann daher beispielsweise einen samenspezifischen Promotor (bevorzugt den Phaseolin-Promotor (US 5504200), den USP- (Baumlein, H. et al., Mol. Gen. Genet. (1991) 225 (3), 459 - 467) oder LEB4-Promotor (Fiedler und Conrad, 1995)), das LEB4-Signalpeptid, das zu exprimierende Gen und ein ER-Retentionssignal enthalten.

Die Herstellung einer Expressionskassette erfolgt durch Fusion eines geeigneten Promotors mit einer geeigneten MPMT-DNA Sequenz und vorzugsweise einer zwischen Promotor und MPMT-DNA-Sequenz inserierten DNA, die für ein chloroplastenspezifisches Transitpeptid kodiert, sowie einem Polyadenylierungssignal nach gängigen Rekombinations- und Klonierungstechniken, wie sie beispielsweise in T. Maniatis, E.F. Fritsch und J. Sambrook, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1989) sowie in T.J. Silhavy, M.L. Berman und L.W. Enquist, Experiments with Gene Fusions, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1984) und in Ausubel, F.M. et al., Current Protocols in Molecular Biology, Greene Publishing Assoc. and Wiley-Interscience (1987) beschrieben sind.

Insbesondere bevorzugt sind Sequenzen, die ein Targeting in den Plastiden gewährleisten.

Es können auch Expressionskassetten verwendet werden, deren DNA-Sequenz für ein MPMT-Fusionsprotein kodiert, wobei ein Teil des Fusionsproteins ein Transitpeptid ist, das die Translokation des Polypeptides steuert. Bevorzugt sind für die Chloroplasten spezifische Transitpeptide, welche nach Translokation des MPMT-Gens in die Chloroplasten vom MPMT-Teil enzymatisch abgespalten werden. Insbesondere bevorzugt ist das Transitpeptid, das von der plastidären Nicotiana tabacum Transketolase oder einem anderen Transitpeptid (z.B. dem Transitpeptid der kleinen Untereinheit der Rubisco oder der Ferredoxin NADP Oxidoreduktase) oder dessen funktionellem Äquivalent abgeleitet ist.

Besonders bevorzugt sind DNA-Sequenzen von drei Kassetten des Plastiden-Transitpeptids der plastidären Transketolase aus Tabak in drei Leserastern als KpnI/BamHI Fragmente mit einem ATG-Codon in der NcoI Schnittstelle:

pTP09

KpnI_GGTACCATGGCGTCTTCTTCTTCTCTCACTCTCTCTCAAGCTATCCTCTCTCGTTCTGTC
CCTCGCCATGGCTCTGCCTCTTCTTCTCAACTTTCCCCTTCTTCTCTCACTTTTTCCGGCCTTAA
ATCCAATCCCAATATCACCACCTCCCGCCGCCGTACTCCTTCCTCCGCCGCCGCCGCCGCCGTCG
TAAGGTCACCGGCGATTCGTGCCTCAGCTGCAACCGAAACCATAGAGAAAACTGAGACTGCGGGA
TCC_BamHI

pTP10

KpnI_GGTACCATGGCGTCTTCTTCTTCTCTCACTCTCTCTCAAGCTATCCTCTCTCGTTCTGTC
CCTCGCCATGGCTCTGCCTCTTCTTCTCAACTTTCCCCTTCTTCTCTCACTTTTTCCGGCCTTAA
ATCCAATCCCAATATCACCACCTCCCGCCGCCGTACTCCTTCCTCCGCCGCCGCCGCCGCCGTCG
TAAGGTCACCGGCGATTCGTGCCTCAGCTGCAACCGAAACCATAGAGAAAACTGAGACTGCGCTG
GATCC_BamHI

pTP11

KpnI_GGTACCATGGCGTCTTCTTCTTCTCTCACTCTCTCTCAAGCTATCCTCTCTCGTTCTGTC
CCTCGCCATGGCTCTGCCTCTTCTTCTCAACTTTCCCCTTCTTCTCTCACTTTTTCCGGCCTTAA
ATCCAATCCCAATATCACCACCTCCCGCCGCCGTACTCCTTCCTCCGCCGCCGCCGCCGCCGTCG
TAAGGTCACCGGCGATTCGTGCCTCAGCTGCAACCGAAACCATAGAGAAAACTGAGACTGCGGGG
ATCC_BamHI

Die inserierte Nukleotid-Sequenz kodierend für eine MPMT kann synthetisch hergestellt oder natürlich gewonnen sein oder eine Mischung aus synthetischen und natürlichen DNA-Bestandteilen enthalten, sowie aus verschiedenen heterologen MPMT-Genabschnitten verschiedener Organismen bestehen. Im allgemeinen werden synthetische Nukleotid-Sequenzen mit Kodons erzeugt, die von Pflanzen bevorzugt werden. Diese von Pflanzen bevorzugten Kodons können aus Kodons mit der höchsten Proteinhäufigkeit bestimmt werden, die in den meisten interessanten Pflanzenspezies exprimiert werden. Bei der Präparation einer Expressionskassette können verschiedene DNA-Fragmente manipuliert werden, um eine Nukleotid-Sequenz zu erhalten, die zweckmäßigerweise in der korrekten Richtung liest und die mit einem korrekten Leseraster ausgestattet ist. Für die Verbindung der DNA-Fragmente miteinander können an die Fragmente Adaptoren oder Linker angesetzt werden.

Zweckmäßigerweise können die Promotor- und die Terminator-Regionen in Transkriptionsrichtung mit einem Linker oder Polylinker, der eine oder mehrere Restriktionsstellen für die Insertion dieser Sequenz enthält, versehen werden. In der Regel hat der Linker 1 bis 10, meistens 1 bis 8, vorzugsweise 2 bis 6 Restriktionsstellen. Im allgemeinen hat der Linker innerhalb der regulatorischen Bereiche eine Größe von weniger als 100 bp, häufig weniger als 60 bp, mindestens jedoch 5 bp. Der Promotor kann sowohl nativ bzw. homolog als auch fremdartig bzw. heterolog zur Wirtspflanze sein. Die Expressionskassette beinhaltet in der 5'-3'-Transkriptionsrichtung den Promotor, eine DNA-Sequenz die für ein MPMT-Gen codiert und eine Region für die transkriptionale Termination. Verschiedene Terminationsbereiche sind gegeneinander beliebig austauschbar.

Ferner können Manipulationen, die passende Restriktionsschnittstellen bereitstellen oder die überflüssige DNA oder Restriktionsschnittstellen entfernen, eingesetzt werden. Wo Insertionen, Deletionen oder Substitutionen wie z.B. Transitionen und Transversionen in Frage kommen, können *in vitro*-Mutagenese, "primerrepair", Restriktion oder Ligation verwendet werden. Bei geeigneten Manipulationen, wie z.B. Restriktion, "chewing-back" oder Auffüllen von Überhängen für "bluntends", können komplementäre Enden der Fragmente für die Ligation zur Verfügung gestellt werden.

Bevorzugte Polyadenylierungssignale sind pflanzliche Polyadenylierungssignale, vorzugsweise solche, die im wesentlichen T-DNA-Polyadenylierungssignale aus Agrobacterium tumefaciens, insbesondere des Gens 3 der T-DNA (Octopin Synthase) des Ti-Plasmids pTiACH5 entsprechen (Gielen et al., EMBO J. 3 (1984), 835 ff) oder funktionelle Äquivalente.

Vorzugsweise wird die fusionierte Expressionskassette, die für ein MPMT-Gen kodiert, in einen Vektor, beispielsweise pBin19, kloniert, der geeignet ist, Agrobacterium tumefaciens zu transformieren. Mit einem solchen Vektor transformierte Agrobakterien können dann in bekannter Weise zur Transformation von Pflanzen, insbesondere von Kulturpflanzen, wie z.B. von Tabakpflanzen, verwendet werden, indem beispielsweise verwundete Blätter oder Blattstücke in einer Agrobakterienlösung gebadet und anschließend in geeigneten Medien kultiviert werden. Die Transformation von Pflanzen durch Agrobakterien ist unter anderem bekannt aus F.F. White, Vectors for Gene Transfer in Higher Plants; in Transgenic Plants, Vol. 1, Engineering and Utilization, herausgegeben von S.D. Kung und R. Wu, Academic Press, 1993, S. 15 - 38. Aus den transformierten Zellen der verwundeten Blätter bzw. Blattstücke können in bekannter Weise transgene Pflanzen regeneriert werden,

die ein in die Expressionskassette integriertes Gen für die Expression eines MPMT-Gens enthalten.

Zur Transformation einer Wirtspflanze mit einer für eine MPMT kodierenden DNA wird eine Expressionskassette als Insertion in einen rekombinanten Vektor eingebaut, dessen Vektor-DNA zusätzliche funktionelle Regulationssignale, beispielsweise Sequenzen für Replikation oder Integration enthält. Geeignete Vektoren sind unter anderem in "Methods in Plant Molecular Biology and Biotechnology" (CRC Press), Kap. 6/7, S. 71 - 119 (1993) beschrieben.

Unter Verwendung der oben zitierten Rekombinations- und Klonierungstechniken können die Expressionskassetten in geeignete Vektoren kloniert werden, die ihre Vermehrung, beispielsweise in E. coli, ermöglichen. Geeignete Klonierungsvektoren sind u.a. pBR332, pUC-Serien, M13mp-Serien und pACYC184. Besonders geeignet sind binäre Vektoren, die sowohl in E. coli als auch in Agrobakterien replizieren können.

Ein weiterer Gegenstand der Erfindung betrifft die Verwendung einer Expressionskassette enthaltend eine DNA-Sequenz SEQ-ID Nr. 1 oder eine mit dieser hybridisierende DNA-Sequenz zur Transformation von Pflanzen, -zellen, -geweben oder Pflanzenteilen. Vorzugsweise ist Ziel der Verwendung die Erhöhung des Gehaltes an Tocopherolen und Tocotrienolen der Pflanze.

Dabei kann je nach Wahl des Promotors die Expression spezifisch in den Blättern, in den Samen, Blütenblättern oder anderen Teilen der Pflanze erfolgen. Solche transgenen Pflanzen, deren Vermehrungsgut, sowie deren Pflanzenzellen, -gewebe oder -teile sind ein weiterer Gegenstand der vorliegenden Erfindung.

Die Expressionskassette kann darüberhinaus auch zur Transformation von Bakterien, Cyanobakterien, Hefen, filamentösen Pilzen und Algen mit dem Ziel einer Erhöhung des Gehaltes an Tocopherolen und Tocotrienolen eingesetzt werden.

Die Übertragung von Fremdgenen in das Genom einer Pflanze wird als Transformation bezeichnet. Es werden dabei die beschriebenen Methoden zur Transformation und Regeneration von Pflanzen aus Pflanzengeweben oder Pflanzenzellen zur transienten oder stabilen Transformation genutzt. Geeignete Methoden sind die Protoplastentransformation durch Polyethylenglykol-induzierte DNA-Aufnahme, das biolistische Verfahren mit der Genkanone - die sogenannte particle bombardment Methode, die Elektroporation, die Inkubation trockener Embryonen in DNA-haltiger Lösung, die Mikroinjektion

(84) **Bestimmungsstaaten** *(regional)*: ARIPO-Patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), eurasisches Patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), europäisches Patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI-Patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Veröffentlicht:

— *Mit internationalem Recherchenbericht.*

— *Vor Ablauf der für Änderungen der Ansprüche geltenden Frist; Veröffentlichung wird wiederholt, falls Änderungen eintreffen.*

Zur Erklärung der Zweibuchstaben-Codes, und der anderen Abkürzungen wird auf die Erklärungen ("Guidance Notes on Codes and Abbreviations") am Anfang jeder regulären Ausgabe der PCT-Gazette verwiesen.

und der durch Agrobacterium vermittelte Gentransfer. Die genannten Verfahren sind beispielsweise in B. Jenes et al., Techniques for Gene Transfer, in: Transgenic Plants, Vol. 1, Engineering and Utilization, herausgegeben von S.D. Kung und R. Wu, Academic Press (1993), 128 - 143 sowie in Potrykus, Annu. Rev. Plant Physiol. Plant Molec. Biol. 42 (1991), 205 - 225) beschrieben. Vorzugsweise wird das zu exprimierende Konstrukt in einen Vektor kloniert, der geeignet ist, Agrobacterium tumefaciens zu transformieren, beispielsweise pBin19 (Bevan et al., Nucl. Acids Res. 12 (1984), 8711).

Mit einer Expressionskassette transformierte Agrobakterien können ebenfalls in bekannter Weise zur Transformation von Pflanzen, insbesondere von Kulturpflanzen, wie Getreide, Mais, Hafer, Soja, Reis, Baumwolle, Zuckerrübe, Canola, Sonnenblume, Flachs, Hanf, Kartoffel, Tabak, Tomate, Raps, Alfalfa, Salat und den verschiedenen Baum-, Nuß- und Weinspezies, verwendet werden, z.B. indem verwundete Blätter oder Blattstücke in einer Agrobakterienlösung gebadet und anschließend in geeigneten Medien kultiviert werden.

Funktionell äquivalente Sequenzen, die für ein MPMT-Gen kodieren, sind solche Sequenzen, welche trotz abweichender Nukleotidsequenz noch die gewünschten Funktionen besitzen. Funktionelle Äquivalente umfassen somit natürlich vorkommende Varianten der hierin beschriebenen Sequenzen sowie künstliche, z.B. durch chemische Synthese erhaltene, an den Kodon-Gebrauch einer Pflanze angepaßte, künstliche Nukleotid-Sequenzen.

Unter einem funktionellen Äquivalent versteht man insbesondere auch natürliche oder künstliche Mutationen einer ursprünglich isolierten für eine MPMT kodierende Sequenz, welche weiterhin die gewünschte Funktion zeigen. Mutationen umfassen Substitutionen, Additionen, Deletionen, Vertauschungen oder Insertionen eines oder mehrerer Nukleotidreste. Somit werden beispielsweise auch solche Nukleotidsequenzen durch die vorliegende Erfindung mit umfaßt, welche man durch Modifikation der MPMT-Nukleotidsequenz erhält. Ziel einer solchen Modifikation kann z.B. die weitere Eingrenzung der darin enthaltenen kodierenden Sequenz oder z.B. auch die Einfügung weiterer Restriktionsenzym-Schnittstellen sein.

Beispiel 8 beschreibt einen Deletionsklon des MPMT-Gens, siehe SEQ-ID Nr. 7)

Funktionelle Äquivalente sind auch solche Varianten, deren Funktion, verglichen mit dem Ausgangsgen bzw. Genfragment, abgeschwächt oder verstärkt ist.

Außerdem sind artifizielle DNA-Sequenzen geeignet, solange sie, wie oben beschrieben, die gewünschte Eigenschaft beispielsweise der Erhöhung des Tocopherol-Gehaltes in der Pflanze durch Überexpression eines MPMT-Gens in Kulturpflanzen vermitteln. Solche artifiziellen DNA-Sequenzen können beispielsweise durch Rückübersetzung mittels Molecular Modelling konstruierter Proteine, die MPMT-Aktivität aufweisen oder durch *in vitro*-Selektion ermittelt werden. Besonders geeignet sind kodierende DNA-Sequenzen, die durch Rückübersetzung einer Polypeptidsequenz gemäß der für die Wirtspflanze spezifischen Kodon-Nutzung erhalten wurden. Die spezifische Kodon-Nutzung kann ein mit pflanzengenetischen Methoden vertrauter Fachmann durch Computerauswertungen anderer, bekannter Gene der zu transformierenden Pflanze leicht ermitteln.

Als weitere geeignete äquivalente Nukleinsäure-Sequenzen sind zu nennen Sequenzen, welche für Fusionsproteine kodieren, wobei Bestandteil des Fusionsproteins ein MPMT-Polypeptid oder ein funktionell äquivalenter Teil davon ist. Der zweite Teil des Fusionsproteins kann z.B. ein weiteres Polypeptid mit enzymatischer Aktivität sein oder eine antigene Polypeptidsequenz mit deren Hilfe ein Nachweis auf MPMT-Expression möglich ist (z.B. myc-tag oder his-tag). Bevorzugt handelt es sich dabei jedoch um eine regulative Proteinsequenz, wie z.B. ein Transitpeptid, das das MPMT-Protein in die Plastiden leitet.

Erhöhung des Gehaltes an Tocopherolen und Tocotrienolen bedeutet im Rahmen der vorliegenden Erfindung die künstlich erworbene Fähigkeit einer erhöhten Biosyntheseleistung dieser Verbindungen durch funktionelle Überexpression eines MPMT-Gens SEQ-ID Nr. 1 oder SEQ-ID Nr. 7 in der Pflanze gegenüber der nicht gentechnisch modifizierten Pflanze für die Dauer mindestens einer Pflanzengeneration.

Dabei kann sowohl der Gehalt an Tocopherolen und Tocotrienolen gesteigert werden. Vorzugsweise wird der Gehalt an Tocopherolen gesteigert. Aber es ist auch möglich unter bestimmten Bedingungen vorzugsweise den Gehalt an Tocotrienolen zu steigern.

Der Biosyntheseort von Tocopherolen beispielsweise ist unter anderem das Blattgewebe, so daß eine blattspezifische Expression des MPMT-Gens sinnvoll ist. Es ist jedoch naheliegend, daß die Tocopherol-Biosynthese nicht auf das Blattgewebe beschränkt sein muß, sondern auch in allen übrigen Teilen der Pflanze - besonders in fetthaltigen Samen - gewebespezifisch erfolgen kann.

Darüberhinaus ist eine konstitutive Expression des exogenen MPMT-Gens von Vorteil. Andererseits kann aber auch eine induzierbare Expression wünschenswert erscheinen.

Die Wirksamkeit der Expression des transgen exprimierten MPMT-Gens kann beispielsweise *in vitro* durch Sproßmeristemvermehrung ermittelt werden. Zudem kann eine in Art und Höhe veränderte Expression des MPMT-Gens und deren Auswirkung auf die Tocopherol-Biosyntheseleistung an Testpflanzen in Gewächshausversuchen getestet werden.

Gegenstand der Erfindung sind außerdem transgene Pflanzen, transformiert mit einer Expressionskassette enthaltend die Sequenz SEQ-ID Nr. 1 oder SEQ-ID Nr. 7 oder eine mit dieser hybridisierende bzw. zur Gesamtsequenz oder zu Teilsequenzen homologen DNA-Sequenz, sowie transgene Zellen, Gewebe, Teile und Vermehrungsgut solcher Pflanzen. Besonders bevorzugt sind dabei transgene Kulturpflanzen, wie z.B. Gerste, Weizen, Roggen, Mais, Hafer, Soja, Reis, Baumwolle, Zuckerrübe, Canola, Sonnenblume, Flachs, Hanf, Kartoffel, Tabak, Tomate, Raps, Alfalfa, Tagetes, Salat und die verschiedenen Baum-, Nuß- und Weinspezies.

Pflanzen im Sinne der Erfindung sind mono- und dikotyle Pflanzen.

Gegenstand der Erfindung sind weiterhin photosynthetisch aktive Organismen transformiert mit einer Expressionskassette enthaltend die Sequenz SEQ-ID Nr. 1 oder SEQ-ID Nr. 7 oder eine mit dieser hybridisierende bzw. zur Gesamtsequenz oder zu Teilsequenzen homologen DNA-Sequenz. Photosynthetisch aktive Organismen sind neben Pflanzen, beispielsweise Cyanobakterien, Moose und Algen.

Da es sich bei diesem Biosyntheseweg um einen ausschließlich plastidär-lokalisierten Stoffwechselweg handelt, bietet er optimale Targetenzyme für die Entwicklung von Inhibitoren. Da sich nach heutigem Stand der Technik kein mit der Synechocystis MPMT identisches oder ähnliches Enzym in humanen und tierischen Organismen befindet, ist davon auszugehen, daß Inhibitoren sehr spezifisch auf Pflanzen wirken sollten.

Wie bereits erwähnt ist die MPMT ein potentielles Target für Herbizide. Um effiziente Hemmstoffe der MPMT finden zu können, ist es notwendig, geeignete Testsysteme, mit denen Inhibitor-Enzym-Bindungsstudien durchgeführt werden können, zur Verfügung zu stellen. Hierzu wird beispielsweise die komplette cDNA-Sequenz der MPMT aus Synechocystis in einen Expressionsvektor (pQE, Qiagen) kloniert und in *E. coli* überexprimiert.

Das mit Hilfe der erfindungsgemäßen Expressionskassette exprimierte MPMT-Protein eignet sich besonders zur Auffindung von für die MPMT spezifischen Hemmstoffen.

Dazu kann die MPMT beispielsweise in einem Enzymtest eingesetzt werden, bei dem die Aktivität der MPMT in An- und Abwesenheit des zu testenden Wirkstoffs ermittelt wird. Aus dem Vergleich der beiden Aktivitätsbestimmungen läßt sich eine qualitative und quantitative Aussage über das Hemmverhalten des zu testenden Wirkstoffes machen.

Mit Hilfe des erfindungsgemäßen Testsystems kann eine Vielzahl von chemischen Verbindungen schnell und einfach auf herbizide Eigenschaften überprüft werden. Das Verfahren gestattet es, reproduzierbar aus einer großen Anzahl von Substanzen gezielt solche mit großer Wirkstärke auszuwählen, um mit diesen Substanzen anschließend weitere, dem Fachmann geläufige vertiefte Prüfungen durchzuführen.

Ein weiterer Gegenstand der Erfindung sind Herbizide, die mit dem oben beschriebenen Testsystem identifizierbar sind.

Durch Überexpression der für eine MPMT kodierenden Gensequenz SEQ-ID Nr. 1 oder SEQ-ID Nr. 7 in einer Pflanze wird eine erhöhte Resistenz gegenüber Inhibitoren der MPMT erreicht. Die derart hergestellten transgenen Pflanzen sind ebenfalls Gegenstand der Erfindung.

Das unter Verwendung der DNA-Sequenz SEQ-ID Nr. 1 oder SEQ-ID Nr. 7 hergestellte MPMT-Protein eignet sich auch zur Durchführung von Biotransformationen zur Bereitstellung größerer Mengen 2,3-Dimethyl-6-phytylhydrochinon. Dabei wird 2-Methyl-6-phytylhydrochinon in Gegenwart des Enzyms MPMT und des Cosubstrats S-Adenosyl-L-Methionin zu 2,3-Dimethyl-6-phytylhydrochinon umgesetzt. Die Biotransformation läßt sich prinzipiell mit ganzen Zellen, die das Enzym MPMT exprimieren oder Zellextrakten aus diesen Zellen oder aber mit aufgereinigter oder hochreiner MPMT in Gegenwart von S-Adenosyl-L-Methionin durchführen.

Weitere Gegenstände der Erfindung sind:

- Verfahren zur Transformation einer Pflanze dadurch gekennzeichnet, daß man Expressionskassetten enthaltend eine DNA-Sequenz SEQ-ID Nr. 1 oder SEQ-ID Nr. 7 oder eine mit dieser hybridisierende, bzw. zur Gesamtsequenz oder zu Teilsequenzen homologen DNA-Sequenz in eine Pflanzenzelle oder Protoplasten

von Pflanzen einbringt und diese zu ganzen Pflanzen regeneriert.

- Verwendung der DNA-Sequenz SEQ-ID Nr. 1 oder SEQ-ID Nr. 7 oder eine mit dieser hybridisierende DNA-Sequenz zur Herstellung von Pflanzen mit erhöhtem Gehalt an Tocopherolen und Tocotrienolen durch Expression einer MPMT DNA-Sequenz in Pflanzen.

Die Erfindung wird durch die nun folgenden Beispiele erläutert, ist aber nicht auf diese beschränkt:

Sequenzanalyse rekombinanter DNA

Die Sequenzierung rekombinanter DNA-Moleküle erfolgte mit einem Laserfluoreszenz-DNA-Sequenzierer der Firma Licor (Vertrieb durch MWG Biotech, Ebersbach) nach der Methode von Sanger (Sanger et al., Proc. Natl. Acad. Sci. USA 74 (1977), 5463 - 5467).

Beispiel 1

Identifizierung einer 2-Methyl-6-phytylhydrochinon-methyltransferase aus Synechocystis spec. PCC 6803.

Die Klonierung und Identifizierung der 2-Methyl-6-phytylhydrochinon-methyltransferase aus Synechocystis spec. PCC 6803 erfolgte folgendermaßen:

Unter Verwendung eines in S-Adenosyl-L-Methionin Methyltransferasen konservierten Sequenzmotivs, welches für die Bindung des S-Adenosyl-L-Methionin (SAM) verantwortlich ist (C.P. Joshi und V.L. Chiang. PMB. 37: 663-374, 1998), wurde eine genomische DNA Datenbank von Synechocystis spec. PCC 6803 durchmustert (Kaneko et al., DNA Res. 34:109-136, 1996). Die bei der Durchmusterung identifizierten hypothetischen Proteine, welche über das SAM-Bindemotiv verfügten, wurden mit den Primärsequenzen der Synechocystis spec. PCC 6803 γ-Tocopherol-methyltransferase (bezeichnet als slr0089) sowie der Arabidopsis thaliana γ-Tocopherol-methyltransferase (David Shintani und Dean DellaPenna. Sience. 282:2098-2100,1998) verglichen.

Dabei konnte ein hypothetisches Protein identifiziert werden (bezeichnet sll0418 SEQ.-ID Nr. 2), welches geringe Übereinstimmung in der Aminosäuresequenz mit den γ-Tocopherol-methyltransferasen aus Synechocystis spec. PCC 6803 und Arabidopsis thaliana aufwies (36% bzw. 28% Identität).

Weitere Untersuchungen der Primärsequenz des hypothetischen Proteins sll0418 belegten das Vorkommen einer putativen prokaryontischen Signalsequenz innerhalb der ersten 20 Aminosäuren (PSIGNAL, PC/GENE™ IntelliGenetics, Inc ©1991). Eine solche Sequenz konnte ebenfalls in der Synechocystis spec. PCC 6803 γ-Tocopherolmethyltransferase (slr0089) identifiziert werden (D. Shintani und D. DellaPenna. Sience. 282:2098-2100,1998) und deutet auf eine identische Lokalisation der beiden Proteine hin.

Das vorhergesagte Molekulargewicht des unprozessierten Proteins beträgt 34,9 kDa und liegt damit in einem Bereich der auch für die Synechocystis spec. PCC 6803 γ-Tocopherolmethyltransferase (David Shintani und Dean DellaPenna, Sience. 282:2098-2100,1998) und der aus Paprikafrüchten gereinigten γ-Tocopherolmethyltransferase (d'Harlingue and Camara, Plastid enzymes of terpenoid biosynthesis: Purification of γ-Tocopherol Methyltransferase from Capsicum Chromoplasts. Journal of Biological Chemistry, Vol. 269 No.28, 15200-152003,1985) ermittelt wurde.

Unter Berücksichtigung der Fakten, schlußfolgerten wir, daß es sich bei dem hypothetischen Protein sll0418 um eine Tocopherolmethyltransferase handeln könnte.

Beispiel 2

Amplifikation und Klonierung der 2-Methyl-6-phytylhydrochinon-methyltransferase aus Synechocystis spec. PCC 6803

Die DNA kodierend für den ORF (open reading frame) sll0418 wurde mittels polymerase chain reaction (PCR) aus Synechocystis spec. PCC 6803 gemäß der Methode nach Crispin A. Howitt (BioTechniques 21:32-34,July 1996) unter Verwendung eines sense spezifischen Primers (sll04185' Seq. Nr. 5) und eines antisense spezifischen Primers (sll04183' Seq. Nr. 6) amplifiziert.

Die PCR Bedingungen waren die folgenden:

Die PCR erfolgte in einem 50µl Reaktionsansatz in dem enthalten war:

-5µl einer Synechocystis spec. PCC 6803 Zellsuspension
-0,2 mM dATP, dTTP, dGTP, dCTP
-1,5 mM $Mg(OAc)_2$
-5µg Rinderserum-Albumin
-40pmol sll04185'
-40pmol sll04183'
-15µl 3,3× rTth DNA Polymerase XLPuffer (PE Applied Biosystems)

-5U rTth DNA Polymerase XL (PE Applied Biosystems)
Die PCR wurde unter folgenden Zyklusbedingungen durchgeführt:
Schritt 1: 5 Minuten 94°C (Denaturierung)
Schritt 2: 3 Sekunden 94°C
Schritt 3: 2 Minuten 58°C (Annealing)
Schritt 4: 2 Minuten 72°C (Elongation)
40 Wiederholungen der Schritte 2-4
Schritt 5: 10 Minuten 72°C (Post-Elongation)
Schritt 6: 4°C (Warteschleife)

Das Amplikon wurde unter Verwendung von Standardmethoden in den PCR Klonierungsvektor pGEM-T (Promega) kloniert. Die Identität des erzeugten Amplikons wurde durch Sequenzierung unter Verwendung des M13F (-40) Primers bestätigt.

Beispiel 3

Erzeugung einer sll0418 Knock out Mutante

Ein DNA Konstrukt zur Erzeugung einer Deletionsmutante des ORF sll0418 in Synechocystis spec. PCC 6803 wurde unter Anwendung von Standard Klonierungstechniken erzeugt.

Der Vektor pGEM-T/sll0418 wurde unter Verwendung des Restriktionsenzyms Ball verdaut. Das Vorhandensein von zwei Ball Schnittstellen innerhalb der sll0418 Sequenz (Position Bp 109 bzw Bp 202) hatte den Verlust eines 93 Bp umfassenden internen Fragmentes zur Folge. In die Ball Schnittstellen des sll0418 ORF wurde die Aminoglycosid-3'Phosphotransferase des Transposons Tn903 kloniert. Dazu wurde das Tn903 als EcoR1 Fragment aus dem Vektor pUC4k (Vieira, J und Messing, J Gene:19, 259-268, 1982) isoliert, die überstehenden Enden des Restriktionsverdaus nach Standardmethoden in glatte Enden überführt und in den Ball geschnittenen Vektor pGEM-T/sll0418 ligiert. Der Ligationsansatz wurde zur Transformation von E.coli Xl1 blue Zellen verwendet. Transformanden wurden durch Verwendung von Kanamycin und Ampicillin selektioniert. Ein rekombinantes Plasmid (pGEM-T/sll0418::tn903) wurde isoliert und zur Transformation von Synechocystis spec. PCC 6803 gemäß der Methode nach Williams (Methods Enzymol. 167:776-778, 1987) eingesetzt.

Synechocystis spec. PCC 6803 Transformanden wurden selektioniert auf Kanamycin haltigem (kan) BG-11 Festmedium (Castenholz, Methods in Enzymology, Seite 68-93, 1988) bei 28°C und 30µmol Photonen × $(m^2 \times s)^{-1}$. Vier unabhängige Knock out Mutanten konnten

nach fünf Selektionsrunden (Passagen von Einzelkolonien auf frisches BG-11kn Medium) erzeugt werden.

Der vollständige Verlust des sll0418 Endogens bzw. der Austausch gegen die rekombinante sll0418::tn903 DNA, wurde durch PCR Analysen bestätigt.

Beispiel 4

Vergleich der Tocopherolproduktion in Synechocystis spec. PCC 6803 Wildtypzellen und den erzeugten Knock out Mutanten des ORF sll0418.

Die auf den BG-11kan Agarmedium kultivierten Zellen der vier unabhängigen Synechocystis spec. PCC 6803 Knock out Mutanten des ORF sll0418 sowie untransformierte Wildtypzellen wurden zum Animpfen von Flüssigkulturen verwendet. Diese Kulturen wurden bei 28°C und 30µmol Photonen × $(m^2 \times s)^{-1}$ (30µE) für ca. 3 Tage kultiviert. Nach Bestimmung der OD_{730} der einzelnen Kulturen, wurde die OD_{730} aller Kulturen durch entsprechende Verdünnungen mit BG-11 (Wildtypen) bzw. BG-11kan (Mutanten) synchronisiert. Diese auf Zelldichte synchronisierten Kulturen wurden zum Animpfen von drei Kulturen pro Mutante bzw. der Wildtypkontrollen verwendet. Die biochemischen Analysen konnten somit unter Verwendung von jeweils drei unabhängig gewachsenen Kulturen einer Mutante und der entsprechenden Wildtypen durchgeführt werden. Die Kulturen wurden bis zu einer optischen Dichte von OD_{730}=0,3 angezogen. Das Medium der Zellkultur wurde durch zweimalige Zentrifugation bei 14000 rpm in einer Eppendorf Tischzentrifuge entfernt. Der daran anschließende Aufschluß der Zellen erfolgte durch viermalige Inkubation im Eppendorfschüttler bei 30°C, 1000rpm in 100% Methanol für 15 Minuten, wobei die jeweils erhaltenen Überstände vereinigt wurden. Weitere Inkubationsschritte ergaben keine weitere Freisetzung von Tocopherolen oder Tocotrienolen.

Um Oxidation zu vermeiden, wurden die erhaltenen Extrakte direkt nach der Extraktion mit Hilfe einer Waters Allience 2690 HPLC-Anlage analysiert. Tocopherole und Tocotrienole wurden über eine reverse Phase Säule(ProntoSil 200-3-C30, Bischoff) mit einer mobilen Phase von 100% Methanol getrennt und anhand von Standards (Merck) identifiziert. Als Detektionssystem diente die Fluoreszenz der Substanzen (Anregung 295nm, Emmision 320 nm), die mit Hilfe eines Jasco Fluoreszensdetektors FP 920 nachgewiesen wurde.

In den Synecchocystis spec. PCC 6803 knock out Mutanten des ORF sll0418 konnten keine Tocopherole und Tocotrienole gefunden werden. Tocopherole und Tocotrienole wurden jedoch in den Synecchocystis spec. PCC 6803 Wildtypzellen gemessen.

Der Verlust der Fähigkeit zur Produktion von Tocopherolen und Tocotrienolen innerhalb der knock out Mutanten des ORF sll0418 im Vergleich zu den Synechocystis spec. PCC 6803 Wildtypzellen zeigt, daß das Gen sll0418 für eine 2-Methyl-6-phytylhydrochinon-methyltransferase kodiert.

Beispiel 5

Funktionelle Charakterisierung der 2-Methyl-6-phytylhydrochinon-methyltransferase aus Synechocystis spec. PCC 6803 durch heterologe Expression in E.coli.

Das hypothetische Protein sll0418 aus Synechocystis spec. PCC 6803 konnte durch funktionelle Expression in E.coli als 2-Methyl-6-phytylhydrochinon-methyltransferase identifiziert werden.

Das aus Synechocystis spec. PCC 6803 amplifizierte Gen sll0418 wurde im korrekten Leserahmen in den Expressionsvektor pQE-30 (Qiagen) subkloniert. Die zur Amplifikation des OFR sll0418 aus Synechocystis spec. PCC 6803 verwendeten Primer sll04185' bzw. sll04183' (Sequenz ID Nr. 5 und 6) waren so konstruiert, daß an das 5' Ende und das 3' Ende des Amplikons BamH1 Restriktionsschnittstellen addiert wurden, siehe Sequenz ID Nr. 3. Das sll0418 Fragment wurde unter Verwendung dieser flankierenden BamH1 Restriktionschnittstellen aus dem rekombinanten Plasmid pGEM-T/sll0418 isoliert und unter Anwendung von Standardmethoden in einen BamHI geschnittenen pQE-30 ligiert. Der Ligationsansatz wurde zur Transformation von M15 E.coli Zellen verwendet und Kanamycin und Ampicillin resistente Transformanden wurden analysiert. Die Kanamycin Resistenz wird durch das in den M15 Zellen enthaltene pREP-4 Plasmid vermittelt. Ein rekombinantes Plasmid (pQE-30/sll0418) welches das sll0418 Fragment in der richtigen Orientierung trug, wurde isoliert. Die Identität und Orientierung des Inserts wurde durch Sequenzierung bestätigt.

Das rekombinante Plasmid pQE-30/sll0418 wurde zur Transformation von M15 E.coli Zellen verwendet, um rekombinantes sll0418 Protein zu erzeugen. Unter Verwendung einer aus der Transformation hervorgegangenen Kolonie wurde eine Übernachtkultur in Luria Broth Medium mit 200µg/ml Ampicillin (Amp) und 50µg/ml Kanamycin (Kan) angeimpft. Ausgehend von dieser Kultur wurde am nächsten

Morgen eine 100ml Luria Broth Kultur (Amp/Kan) angeimpft. Diese Kultur wurde bei 28°C auf einem Schüttelinkubator bis zum erreichen einer OD_{600}:0,35-0,4 inkubiert. Anschließend wurde die Produktion des rekombinanten Proteins durch Zugabe von 0,4 mM Isopropyl-ß-D-thiogalaktopyranosid (IPTG) induziert. Die Kultur wurde für weitere 3 Stunden bei 28°C geschüttelt und die Zellen anschließend durch Zentrifugation bei 8000g pelletiert.

Das Pellet wurde in 600µl Lysispuffer (ca. 1-1,5 ml /g Pellet Naßgewicht, 10 mM HEPES KOH pH 7,8, 5 mM Dithiothreitol (DTT), 0,24 M Sorbitol) resuspendiert. Anschließend wurde PMSF (Phenylmethylsulfonat) zu einer Endkonzentration von 0,15 mM beigefügt und der Ansatz für 10 Minuten auf Eis gestellt. Der Aufschluß der Zellen erfolgte durch einen 10 Sekunden Ultraschall-Puls unter Verwendung eines Ultraschallstabes. Nach Zugabe von Triton X100 (Endkonzentration 0,1%) wurde die Zellsuspension für 30 Minuten auf Eis inkubiert. Der Ansatz wurde anschließend für 30 Minuten bei 25000xg abzentrifugiert und der Überstand zum Assay eingesetzt.

Die Aktivitätsbestimmung der 2-Methyl-6-phytylhydrochinon-methyltransferase erfolgt durch Nachweis des radioaktiv markierten Reaktionsproduktes 2,3-Dimethyl-6-phytylhydrochinon.

Dazu wurden 135µl des Enzyms (ca.300-600µg) zusammen mit 20µl Substrat (2-Methyl-6-phytylhydrochinon) und 15µl (0,46 mM SAM ^{14}C) Methylgruppendonor in folgendem Reaktionspuffer : 200µl (125mM) Tricine-NaOH pH 7,6, 100µl (1,25 mM) Sorbitol, 10µl (50mM) $MgCl_2$ und 20µl (250mM) Ascorbat für 4 Stunden bei 25°C im Dunkeln inkubiert.

Das Abstoppen der Reaktion erfolgte durch Zugabe von 750µl Chloroform/Methanol (1:2) + 150µl 0,9% NaCl. Der gemischte Ansatz wurde kurz zentrifugiert und die obere Phase wurde verworfen. Die untere Phase wird in ein neues Reaktionsgefäß überführt und unter Stickstoff eingedampft. Die Rückstände wurden in 20µl Ether aufgenommen und auf eine Dünnschicht-Platte zur chromatographischen Trennung der Substanzen aufgetragen (feste Phase: HPTLC-Platten: Kieselgel 60 F_{254} (Merk), flüssige Phase: Toluol). Der Nachweis des radioaktiv markierten Reaktionsproduktes erfolgt durch Verwendung eines Phosphoimagers.

Diese Experimente bestätigten, daß es sich bei dem durch das Gen s110418 (SEQ-ID Nr.1) aus Synechocystis spec. PCC 6803 kodierte Protein um eine 2-Methyl-6-phytylhydrochinon-methyltransferase handelt, da es die enzymatische Aktivität zur Umwandlung von

2-Methyl-6-phytylhydrochinon in 2,3-Dimethyl-6-phytylhydrochinon besitzt.

Abbildung 2 zeigt einen Sequenzvergleich auf Aminosäureebene zwischen den γ-Tocopherolmethyltransferasen aus Synechocystis spec. PCC Synechocystis spec. PCC 6803 (slr0089) und *A.thaliana* (aratmt) mit der 2-Methyl-6-phytylhydrochinon-methyltransferase (sll04189) aus Synechocystis spec. PCC 6803. Die Übereinstimmung mit den γ-Tocopherolmethyltransferasen aus Synechocystis spec. PCC 6803 und Arabisopsis thaliana beträgt 36 bzw. 28 % Identität.

Beispiel 6

Substratspezifität der 2-Methyl-6-phytylhydrochinon-methyltransferase

Enzymatische Untersuchungen wie in Beispiel 5 durchgeführt belegen, daß das Enzym MPMT - kodiert durch das Gen sll0418 (SEQ-ID Nr. 1) aus Synechocystis spec. PCC 6803 - 2-Methyl-6-phytylhydrochinon in 2,3-Dimethyl-6-phytylhydrochinon umwandelt.

Zusätzlich besitzt das Enzym MPMT eine 2-Methyl-6-geranylgeranylhydrochinon-methyltransferase Aktivität, wohingegen eine γ-Tocopherolmethyltransferase Aktivität nicht nachgewiesen werden konnte. Somit ist belegt, daß das Enzym 2-Methyl-6-phytylhydrochinon-methyltransferase an der Biosynthese der Tocotrienole beteiligt ist, da es 2-Methyl-6-geranylgeranylhydrochinon zu 2,3-Dimethyl-6-geranylgeranyl-hydrochinon umwandelt. Dies zeigt deutlich die Verschiedenheit der Enzymaktivität der 2-Methyl-6-phytylhydrochinon-methyltransferase im Vergleich zur γ-Tocopherolmethyltransferase.

Beispiel 7

Herstellung von Expressionskassetten enthaltend das MPMT-Gen

Transgene Pflanzen wurden erzeugt, die die 2-Methyl-6-phytylhydrochinon-methyltransferase aus Synechocystis spec. PCC6803 zum einen unter Kontrolle des konstitutiven 35S-Promotor des CaMV (Blumenkohlmosaikvirus) (Franck et al., Cell 21: 285-294, 1980) und zum anderen unter Kontrolle des samenspezifischen Promotors des Legumin Gens aus Vicia faba (Kafatos et al., Nuc. Acid. Res.,14(6): 2707-2720, 1986) exprimieren. Die Grundlage des zur konstitutiven Expression der 2-Methyl-6-phytylhydrochinon-methyltransferase aus Synechocystis spec. PCC 6803 erzeugten Plasmides war der pBinAR-TkTp-9 (Ralf Badur , Dissertation Universität Göttingen, 1998). Dieser Vektor ist ein Derivat des pBinAR (Höfgen

und Willmitzer, Plant Sci. 66: 221-230, 1990) und enthält den 35S-Promotor des CaMV (Blumenkohlmosaikvirus) (Franck et al., 1980), das Terminationssignal des Octopin-Synthase Gens (Gielen et al., EMBO J. 3: 835-846, 1984) sowie die für das Transitpeptid der plastidären Nicotiana tabacum Transketolase kodierende DNA Sequenz (Ralf Badur, Dissertation Universität Göttingen, 1998). Die unter Berücksichtigung des korrekten Leserasters erfolgte Klonierung der 2-Methyl-6-phytylhydrochinon-methyltransferase aus Synechocystis spec. PCC6803 in diesen Vektor, erzeugt eine Translationsfusion der 2-Methyl-6-phytylhydrochinon-methyltransferase mit dem plastidären Transitpeptid. Dadurch erfolgt ein Transport des Transgens in die Plastiden.

Zur Erstellung dieses Plasmides wurde das Gen sll0418 unter Verwendung der flankierenden BamHI Restriktionsschnittstellen aus dem Plasmid pGEM-T/sll0418 isoliert. Dieses Fragment wurde unter Anwendung von Standardmethoden in einen BamHI geschnittenen pBinAR-TkTp-9 ligiert (siehe Abbildung 3). Dieses Plasmid (pBinAR-TkTp-9/sll0418) wurde zur Erzeugung transgener Arabidopsis thaliana, Brassica napus und Nicotiana tabacum verwendet. Fragment A (529 bp) in Abbildung 3 beinhaltet den 35S-Promotor des CaMV (Nukleotide 6909 bis 7437 des Blumenkohlmosaikvirus), Fragment B (245bp) kodiert für das Transitpeptid der Nicotiana tabacum Transketolase, Fragment C (977Bp) kodiert ORF sll0418 aus Synechocystis spec. PCC 6803, Fragment D (219Bp) kodiert für das Terminationssignal des Octopin-Synthase Gens.

Zur Erzeugung eines Plasmides, welches die samenspezifische Expression der 2-Methyl-6-phytylhydrochinon-methyltransferase aus Synechocystis spec. PCC 6803 in Pflanzen ermöglicht, wurde der samenspezifiche Promotor des Legumin B4 Gens (Kafatos et al., Nuc. Acid. Res.,14(6):2707-2720, 1986) verwendet. Aus dem Plasmid pCR-Script/lePOCS wurde das 2,7 Kb Fragment des Legumin B4 Gen Promotors unter Verwendung der den Promotor 5' flankierenden EcoR1 und der 3' flankierenden Kpn1 Schnittstellen isoliert. Das Plasmid pBinAR-TkTp-9/sll0418 wurde ebenfalls mit den Restriktionsenzymen EcoR1 und Kpn1 behandelt. Dies hatte zur Folge, daß der 35S-Promotor des CaMV aus diesem Plasmid herausgetrennt wurde. Der Promotor des Legumin Gens wurde anschließend als EcoR1/Kpn1 Fragment in diesen Vektor kloniert, wodurch ein Plasmid erzeugt wurde, welches die Expression des Gen sll0418 unter die Kontrolle dieses samenspezifischen Promotors stellte, siehe Abbildung 4. Dieses Plasmid (pBinARleP-TkTp-9/sll0418) wurde zur Erzeugung transgener Arabidopsis thaliana, Brassica napus und Nicotiana tabacum Pflanzen verwendet.

Fragment A (2700 bp) in Abbildung 4 beinhaltet den Promotor des Legumin B4 Gens aus Vicia faba, Fragment B (245bp) kodiert für das Transitpeptid der Nicotina tabacum Transketolase, Fragment C (977Bp) kodiert für das ORF sll0418 aus Synechocystis spec. PCC 6803 , Fragment D (219Bp) für das Terminationssignal des Octopin-Synthase Gens.

Beispiel 8

Herstellung von Expressionskassetten enthaltend einen Deletionsklon des MPMT-Gens

Auf Grundlage einer Computeranalyse wurde in der Primärsequenz des ORF sll0418 ein putatives prokaryontisches Sekretionssignal identifiziert. Um sicherzustellen, daß dieses bei der Expression in Pflanzen keinen negativen Einfluß auf den Import des Proteins in die Plastiden nimmt, wurde ein Derivat der Sequenz des sll0418 erzeugt, bei dem das putative Sekretionssignal deletiert wurde (Sequenz-ID Nr. 7). Diese Deletion wurde unter Anwendung der PCR Technologie durchgeführt. Durch die dabei verwendeten Primer (sll0418DSp5', Sequenz-ID Nr. 9 und sll0418DSp3',Sequenz-ID Nr. 10) wurde an das 5'Ende der Sequenz eine EcoRV Restriktionsschnittstelle und an das 3'Ende eine SalI Restriktionsschnittstelle addiert, durch die eine gerichtete Klonierung in den Vektor pBinAR-TkTp-9 ermöglicht wurde. Das entstandene Plasmid pBinAR-TkTp-9/sll0418ΔSP ist in Abbildung 5 beschrieben. Fragment A (529 bp) in Abbildung 5 beinhaltet den 35S-Promotor des CaMV (Nukleotide 6909 bis 7437 des Blumenkohlmosaikvirus), Fragment B (245bp) Fragment kodiert für das Transitpeptid der Nicotiana tabacum Transketolase, Fragment C (930Bp) ORF sll0418ΔSP aus Synechocystis spec. PCC 6803 Fragment D (219Bp) für das Terminationssignal des Octopin-Synthase Gens.

Zur Erzeugung eines Plasmides, welches die samenspezifische Expression des Deletionsklons der 2-Methyl-6-phytylhydrochinon-methyltransferase aus Synechocystis spec. PCC6803 in Pflanzen ermöglicht, wurde ebenfalls der bereits beschriebene samenspezifische Promotor des Legumin B4 Gens (Kafatos et al., Nuc. Acid. Res.,14(6):2707-2720, 1986) verwendet. Aus dem Plasmid PCR-Script/lePOCS wurde das 2,7 Kb Fragment des Legumin B4 Gen Promotors unter Verwendung der den Promotor 5' flankierenden EcoR1 und der 3' flankierenden Kpn1 Schnittstellen isoliert. Das Plasmid pBinAR-TkTp-9/sll0418ΔSP wurde ebenfalls mit den Restriktionsenzymen EcoR1 und Kpn1 behandelt. Dies hatte zur Folge, daß der 35S-Promotor des CaMV aus diesem Plasmid herausgetrennt wurde. Der Promotor des Legumin Gens wurde anschließend als EcoR1/Kpn1 Fragment in diesen Vektor kloniert, wodurch ein

Plasmid erzeugt wurde, welches die Expression des Deletionsklons des Gen sll0418 unter die Kontrolle dieses samenspezifischen Promotors stellte, siehe Abbildung 6. Fragment A (2700 bp) in Abbildung 6 beinhaltet den Promotor des Legumin B4 Gens aus Vicia faba, Fragment B (245bp) Fragment kodiert für das Transitpeptid der Nicotiana tabacum Transketolase, Fragment C (930Bp) ORF sll0418ΔSP aus Synechocystis spec. PCC 6803 Fragment D (219Bp) für das Terminationssignal des Octopin-Synthase Gens.

Dieses Plasmid (pBinARleP-TkTp-9/sll0418ΔSP) wurde zur Erzeugung transgener Arabidopsis thaliana, Brassica napus und Nicotiana tabacum Pflanzen verwendet.

Auch durch Expression der DNA-Sequenz SEQ-ID Nr. 7 in transgenen Pflanzen wurde eine Steigerung des Gehaltes an Tocopherol und Tocotrienol gemessen.

Beispiel 9

Herstellung transgener Arabidopsis thaliana Pflanzen

Wildtyp Arabidopsis thaliana Pflanzen (Columbia) wurden mit dem Agrobacterium tumefaciens Stamm (EHA105) auf Grundlage einer modifizierten Vacuum Infiltrationsmethode transformiert (Steve Clough und Andrew Bent, Floral dip: a simplified method for Agrobacterium mediated transformation of Arabidopsis thaliana. Plant J. 16(6):735-43, 1998; Bechtold, N., Ellis, J. und Pelltier, G., in: Planta Agrobacterium-mediated gene transfer by infiltration of adult Arabidopsis thaliana plants. CRAcad Sci Paris, 1993. 1144(2):204-212). Die verwendeten Agrobacterium tumefaciens Zellen waren im Vorfeld mit den Plasmiden pBinARleP-TkTp-9/sll0418 bzw. pBinAR-TkTp-9/sll0418 (Abbildung 3 und 4) transformiert worden.

Samen der Primärtransformanden wurden auf Grundlage der Antibiotikaresistenz selektioniert. Antibiotika resistente Keimlinge wurden in Erde gepflanzt und als vollentwickelte Pflanzen zur biochemischen Analyse verwendet.

Beispiel 10

Herstellung transgener Brassica napus Pflanzen

Die Herstellung transgener Raps Pflanzen orientierte sich an einem Protokoll von Bade, J.B. und Damm,B. (in Gene Transfer to Plants, Potrykus, I. und Spangenberg, G., eds, Springer Lab Ma-

nual, Springer Verlag, 1995, 30-38), in welchem auch die Zusammensetzung der verwendeten Medien und Puffer angegeben ist.

Die Transformationen erfolgten mit dem Agrobacterium tumefaciens Stamm EHA105. Zur Transformation wurden die Plasmide pBinARleP-TkTp-9/s110418 bzw. pBinAR-TkTp-9/s110418 verwendet. Samen von Brassica napus var. Westar wurden mit 70% Ethanol (v/v) oberflächensteril gemacht, 10 Minuten bei 55°C in Wasser gewaschen, in 1%iger Hypochlorit-Lösung (25% v/v Teepol,0,1% v/v Tween 20) für 20 Minuten inkubiert und sechsmal mit sterilem Wasser für jeweils 20 Minuten gewaschen. Die Samen wurden drei Tage auf Filterpapier getrocknet und 10-15 Samen in einem Glaskolben mit 15 ml Keimungsmedium zur Keimung gebracht. Von mehreren Keimlingen (ca. 10 cm groß) wurden die Wurzeln und Apices entfernt und die verbleibenden Hypokotyle in ca. 6 mm lange Stücke geschnitten. Die so gewonnenen ca. 600 Explantate wurden 30 Minuten mit 50 ml Basalmedium gewaschen und in einen 300 ml Kolben überführt. Nach Zugabe von 100 ml Kallusinduktionsmedium wurden die Kulturen für 24 Stunden bei 100 U/min inkubiert.

Vom Agrobacterium Stamm wurde eine Übernachtkultur bei 29°C in Luria Broth-Medium mit Kanamycin (20mg/l) angesetzt, davon 2 ml in 50 ml Luria Broth-Medium ohne Kanamycin für 4 Stunden bei 29°C bis zu einer OD_{600} von 0,4-0,5 inkubiert. Nach der Pelletierung der Kultur bei 2000 U/min für 25 min wurde das Zellpellet in 25 ml Basalmedium resuspendiert. Die Konzentration der Bakterien in der Lösung wurde durch Zugabe von weiterem Basalmedium auf eine OD_{600} von 0,3 eingestellt.

Aus den Raps-Explanten wurde das Kallus-Induktionsmedium mit sterilen Pipetten entfernt, 50 ml Agrobacterium-Lösung hinzugefügt, vorsichtig gemischt und für 20 min inkubiert. Die Agrobacterien-Suspension wurde entfernt, die Raps-Explante für 1 min mit 50 ml Kallus-Induktionsmedium gewaschen und anschließend 100 ml Kallus-Induktionsmedium hinzugefügt. Die Co-Kultivierung wurde für 24 h auf einem Rotationsschüttler bei 100 U/min durchgeführt. Die Co-Kultivierung wurde durch Wegnahme des Kallus-Induktionsmediums gestoppt und die Explante zweimal für jeweils 1 min mit 25 ml und zweimal für 60 min mit jeweils 100 ml Waschmedium bei 100 U/min gewaschen. Das Waschmedium mit den Explanten wurde in 15 cm Petrischalen überführt und das Medium mit sterilen Pipetten entfernt.

Zur Regeneration wurden jeweils 20-30 Explante in 90 mm Petrischalen überführt, welche 25 ml Sproß-Induktionsmedium mit Kanamycin enthielten. Die Petrischalen wurden mit 2 Lagen Leukopor verschlossen und bei 25 °C und 2000 lux bei Photoperioden von 16

Stunden Licht/ 8 Stunden Dunkelheit inkubiert. Alle 12 Tage wurden die sich entwickelnden Kalli auf frische Petrischalen mit Sproß-Induktionsmedium umgesetzt. Alle weiteren Schritte zur Regeneration ganzer Pflanzen wurden wie von Bade, J.B und Damm, B. (in: Gene Transfer to Plants, Potrykus, I. und Spangenberg, G., eds, Springer Lab Manual, Springer Verlag, 1995, 30-38) beschrieben durchgeführt.

Beispiel 11

Herstellung transgener Nicotiana tabacum Pflanzen

Zehn ml YEB-Medium mit Antibiotikum (5 g/l Rinder-Extrakt, 1 g/l Hefe-Extrakt, 5 g/l Pepton, 5 g/l Saccharose und 2 mM $MgSo_4$) wurden mit einer Kolonie von Agrobacterium tumefaciens beimpft und über Nacht bei 28°C kultiviert. Die Zellen wurden 20 min bei 4°C, 3500 U/min in einer Tischzentrifuge pelletiert und danach in frischem YEB-Medium ohne Antibiotika unter sterilen Bedingungen resuspendiert. Die Zellsuspension wurde für die Transformation eingesetzt.

Die Wildtyp-Pflanzen aus Sterilkultur wurden durch vegetative Replikation erhalten. Dazu wurde nur die Spitze der Pflanze abgeschnitten und auf frisches 2MS-Medium in ein steriles Einweckglas überführt. Vom Rest der Pflanze wurden die Haare auf der Blattoberseite und die Mittelrippen der Blätter entfernt. Die Blätter wurden mit einer Rasierklinge in etwa 1 cm^2 große Stücke geschnitten. Die Agrobakterienkultur wurde in eine kleine Petrischale überführt (Durchmesser 2 cm). Die Blattstücke wurden kurz durch die Lösung gezogen und mit der Blattunterseite auf 2MS-Medium in Petrischalen (Durchmesser 9 cm) gelegt, so daß sie das Medium berührten. Nach zwei Tagen im Dunkeln bei 25°C wurden die Explantate auf Platten mit Kallusinduktionsmedium überführt und in der Klimakammer auf 28°C temperiert. Das Medium mußte alle 7-10 Tage gewechselt werden. Sobald sich Kalli bildeten, wurden die Explantate in sterile Einweckgläser auf Sproßinduktionsmedium mit Claforan (siehe oben) überführt. Nach etwa einem Monat trat Organogenese ein und die gebildeten Sprossen konnten abgeschnitten werden. Die Kultivierung der Sprosse wurde auf 2MS-Medium mit Claforan und Selektionsmarker durchgeführt. Sobald sich ein kräftiger Wurzelballen gebildet hatte, konnte die Pflanzen in Pikiererde getopft werden.

Beispiel 12

Charakterisierung der transgenen Pflanzen

Um zu bestätigen, daß durch die Expression der 2-Methyl-6-phytyl-hydrochinon-methyltransferase aus Synechocystis spec. PCC 6803 die Vitamin E Biosynthese in den transgenen Pflanzen gesteigert wird, wurden die Tocopherol- und Tocotrienol-Gehalte in Blätter und Samen der mit den Konstrukten pBinARleP-TkTp-9/s110418 bzw. pBinAR-TkTp-9/s110418 Pflanzen (Arabidopsis thaliana, Brassica napus und Nicotiana tabacum) analysiert. Dazu wurden die transgenen Pflanzen im Gewächshaus kultiviert und Pflanzen die das Gen kodierend für die 2-Methyl-6-phytylhydrochinon-methyltransferase aus Synechocystis spec. PCC 6803 exprimieren auf Northern-Ebene analysiert. In Blättern und Samen dieser Pflanzen wurde der Tocopherolgehalt und der Tocotrienolgehalt ermittelt. In allen Fällen war die Tocopherol- bzw. Tocotrienol-Konzentration in transgenen Pflanzen, die zusätzlich eine DNA-Sequenz SEQ-ID Nr. 1 oder SEQ-ID Nr. 7 exprimieren, im Vergleich zu nicht transformierten Pflanzen erhöht.

Patentansprüche

1. DNA-Sequenz SEQ-ID Nr. 1 oder SEQ-ID Nr. 7 und mit dieser hybridisierende oder zur Gesamtsequenz oder zu Teilsequenzen homologen DNA-Sequenz kodierend für eine 2-Methyl-6-phytylhydrochinon-methyltransferase aus Synechocystis.

2. Verwendung von DNA-Sequenzen codierend für eine 2-Methyl-6-phytylhydrochinon-methyltransferase zur Herstellung von Pflanzen und photosynthetisch aktiven Organismen mit erhöhtem Gehalt an Tocopherolen und Tocotrienolen.

3. Verwendung einer DNA-Sequenz SEQ-ID Nr. 1 oder SEQ-ID Nr. 7 oder einer mit dieser hybridisierenden DNA-Sequenz kodierend für eine 2-Methyl-6-phytylhydrochinon-methyltransferase zur Herstellung von Pflanzen und photosynthetisch aktiven Organismen mit erhöhtem Gehalt an Tocopherolen und Tocotrienolen.

4. Verfahren zur Herstellung von Pflanzen und photosynthetisch aktiven Organismen mit erhöhtem Gehalt an Tocopherolen und Tocotrienolen dadurch gekennzeichnet, daß eine DNA-Sequenz SEQ-ID Nr. 1 oder SEQ-ID Nr. 7 oder eine mit dieser hybridisierende oder zur Gesamtsequenz oder zu Teilsequenzen homologen DNA-Sequenz in Pflanzen und photosynthetisch aktiven Organismen exprimiert wird.

5. Verfahren zur Transformation einer Pflanze dadurch gekennzeichnet, daß man eine Expressionskassette enthaltend einen Promotor, eine Signalsequenz, eine DNA-Sequenz SEQ-ID Nr. 1 oder SEQ-ID Nr. 7 und einen Terminator oder eine mit dieser hybridisierende DNA-Sequenz in eine Pflanzenzelle, in Kallusgewebe, eine ganze Pflanze oder Protoplasten von Pflanzenzellen einbringt.

6. Verfahren zur Transformation von Pflanzen gemäß Anspruch 5, dadurch gekennzeichnet, daß die Transformation mit Hilfe des Stammes Agrobacterium tumefaciens, der Elektroporation oder der particle bombardment Methode erfolgt.

7. Pflanze mit erhöhtem Gehalt an Tocopherolen und Tocotrienolen enthaltend eine Expressionskassette gemäß Anspruch 5.

Zeichn.

8. Pflanze nach Anspruch 7, ausgewählt aus der Gruppe Soja, Canola, Gerste, Hafer, Weizen, Raps, Mais, Roggen, Tagetes oder Sonnenblume.

9. Verwendung der DNA-Sequenz SEQ-ID Nr. 1 oder SEQ-ID Nr. 7 oder einer mit dieser hybridisierende DNA-Sequenz gemäß Anspruch 1 zur Herstellung eines Testsystems zur Identifizierung von Inhibitoren der 2-Methyl-6-phytylhydrochinon-methyltransferase.

10. Testsystem basierend auf der Expression der DNA-Sequenz SEQ-ID Nr. 1 oder SEQ-ID Nr. 7 oder einer mit dieser hybridisierende DNA-Sequenz gemäß Anspruch 1 zur Identifizierung von Inhibitoren der 2-Methyl-6-phytylhydrochinonmethyltransferase.

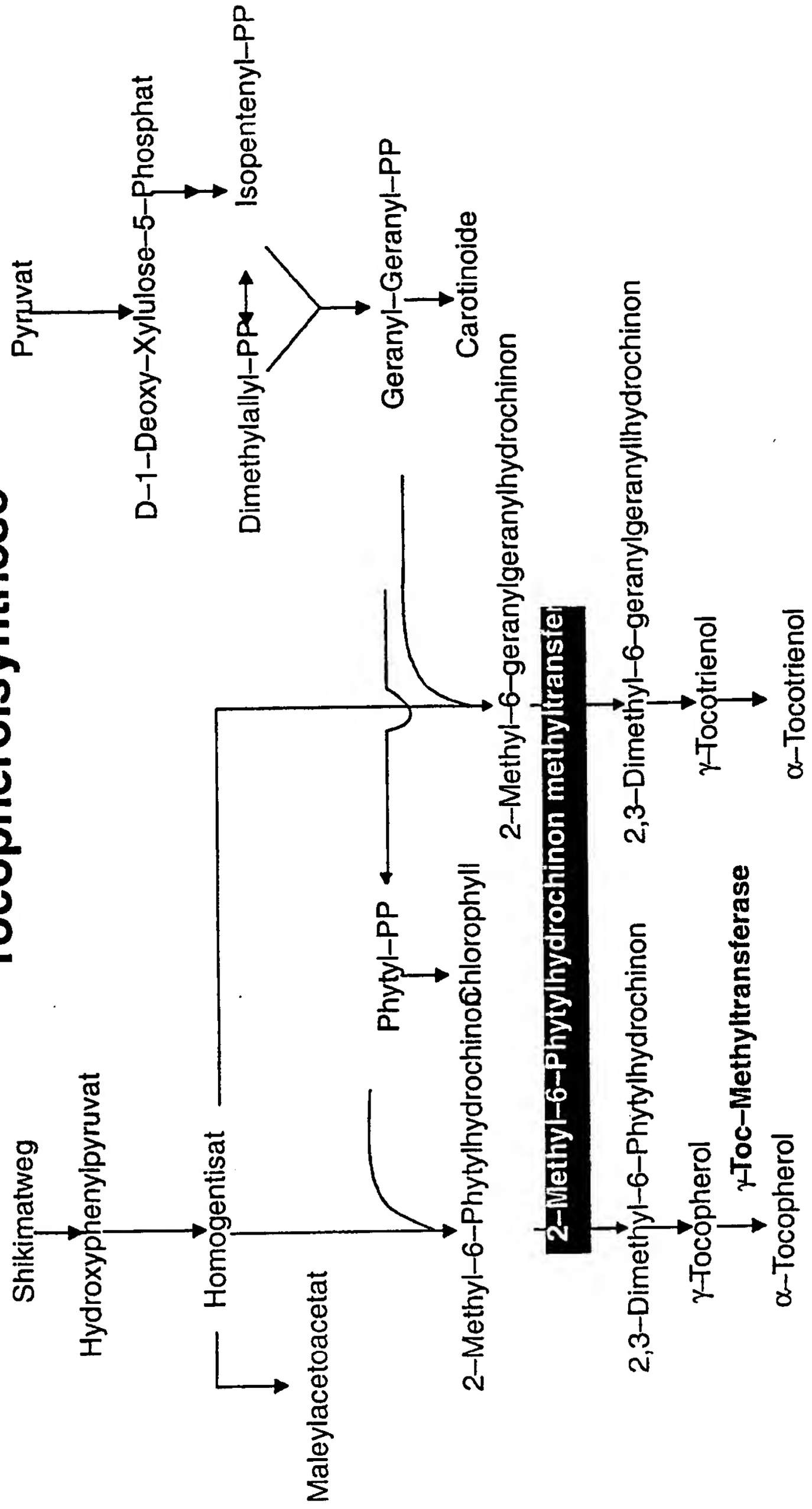

Tocopherolsynthese
Pyruvat
D–1–Deoxy–Xylulose–5–Phosphat
Isopentenyl–PP
Dimethylallyl–PP
Geranyl–Geranyl–PP
Carotinoide
Phytyl–PP
Chlorophyll
2–Methyl–6–geranylgeranylhydrochinon
2–Methyl–6–Phytylhydrochinon methyltransferase
2,3–Dimethyl–6–geranylgeranylhydrochinon
γ–Tocotrienol
α–Tocotrienol
Shikimatweg
Hydroxyphenylpyruvat
Homogentisat
Maleylacetoacetat
2–Methyl–6–Phytylhydrochinon
2–Methyl–6–Phytylhydrochinon methyltransferase
2,3–Dimethyl–6–Phytylhydrochinon
γ–Tocopherol
γ–Toc–Methyltransferase
α–Tocopherol

Abbildung 1

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      - - - - - - - - - - - - - - - - - - - - - - - - - - - M X X X X X X X X X L X S   Majority
                          10                  20                  30                  40
1     - - - - - - - - - - - - - - - - - - - - - - - - - - - M P E Y L L L P A G L I S   sll0418.PRO
1     - - - - - - - - - - - - - - - - - - - - - - - - - - - M V Y H V R P K H A L F L   slr0089.PRO
1     M K A T L A A P S S L T S L P Y R T N S S F G S K S S L L F R S P S S S S S V S   aratmt.PRO

      X X X X X X X X L X X A X A T X X X A X X X X X X X X X X X T S X G X X E X X W   Majority
                          50                  60                  70                  80
14    L S L A I A A G L Y L L T A R G Y Q S S D S V A N A Y D Q W T E D G I L E Y Y W   sll0418.PRO
14    A F Y C Y F S L L T M A S A T I A S A D L Y E K I K N F Y D D S S G L W E D V W   slr0089.PRO
41    M T T T R G N V A V A A A A T S T E A L R K G I A E F Y N E T S G L W E E I W G   aratmt.PRO

      G X H X H X G Y Y G D X X V X X X D - - X X X A Q I X X I X E X L A X A G X X D   Majority
                          90                 100                 110                 120
54    G D H I H L G H Y G D P P V A K - - - - D F I Q S K I D F V H A M A Q W G G L D   sll0418.PRO
54    G E H M H H G Y Y G P H G T Y R I D - - R R Q A Q I D L I K E L L A W A V P Q N   slr0089.PRO
81    D H M H H G F Y D P D S S V Q L S D S G H K E A Q I R M I E E S L R F A G V T D   aratmt.PRO

      X X - - X K X X K V L D V G C G I G G S S R Y L A X X X G A E V X G I T L S P V   Majority
                         130                 140                 150                 160
90    T L - - P P G T T V L D V G C G I G G S S R I L A K D Y G F N V T G I T I S P Q   sll0418.PRO
92    S A - - - K P R K I L D L G C G I G G S S L Y L A Q Q H Q A E V M G A S L S P V   slr0089.PRO
121   E E E E K K I K K V V D V G C G I G G S S R Y L A S K F G A E C I G I T L S P V   aratmt.PRO
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                Q V K R A X E L A X A X X L X X T A X F Q V A D A L D L P F X D G S F D X V W S  Majority
                        170                 180                 190                 200
128 Q V K R A T E L T P P - - - D V T A K F A V D D A M A L S F P D G S F D V V W S  s110418.PRO
129 Q V E R A G E R A R A L G L G S T C Q F Q V A N A L D L P F A S D S F D W V W S  slr0089.PRO
161 Q A K R A N D L A A A Q S L S H K A S F Q V A D A L D Q P F E D G K F D L V W S  aratmt.PRO

                X E S G E H M P D K A X F X K E L X R V X K P G G R L I X A T W C H R X X X X G  Majority
                        210                 220                 230                 240
165 V E A G P H M P D K A V F A K E L L R V V K P G G I L V V A D W N Q R D D R Q V  s110418.PRO
169 L E S G E H M P N K A Q F L Q E A W R V L K P G G R L I L A T W C H R P I D P G  slr0089.PRO
201 M E S G E H M P D K A K F V K E L V R V A A P G G R I I I V T W C H R N L S A G  aratmt.PRO

                X X X L X X X E X X X L X X I X X X X X L P A X X S X X D Y X X X A X X X X X G  Majority
                        250                 260                 270                 280
205 P L N F W E K P V M R Q L L D Q W S H P A F A S I E G F A E N L E A T G L V E G  s110418.PRO
209 N G P L T A D E R R H L Q A I Y D V Y C L P Y V V S L P D Y E A I A R E C G F G  slr0089.PRO
241 E E A L Q P W E Q N I L D K I C K T F Y L P A W C S T D D Y V N L L Q S H S L Q  aratmt.PRO

                X I K T A D W S V X V A P F W X X V I X X A X X X X X L W X L X X X G X K I I X  Majority
                        290                 300                 310                 320
245 Q V T T A D W T V P T L P A W L D T I W Q G I I R P Q G W L Q Y G I R G F I K S  s110418.PRO
249 E I K T A D W S V A V A P F W D R V I E S A F D P R V L W A L G Q A G P K I I N  slr0089.PRO
281 D I K C A D W S E N V A P F W P A V I R T A L T W K G L V S L L R S G M K S I K  aratmt.PRO
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                              330       340       350       360
     X A L X X X L M X X G Y X X - - G L X R F - - G X X T X X K P L X X - X - - - -  Majority
285  V R E V P T I L L M R L A F G V G L C R F - - G M F K A V R K N A T Q A          sll0418.
289  A A L C L R L M K W G Y E R - - G L V R F - - G L L T G I K P L V - . S P Q S P  slr0089.
321  G A L T M P L M I E G Y K K - - G V I K F - - G I I T C Q K P L                  aratmt.l

     -                                                                                Majority

318                                                                                   sll0418.
324  R                                                                                slr0089.
348                                                                                   aratmt.l
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Abbildung 3

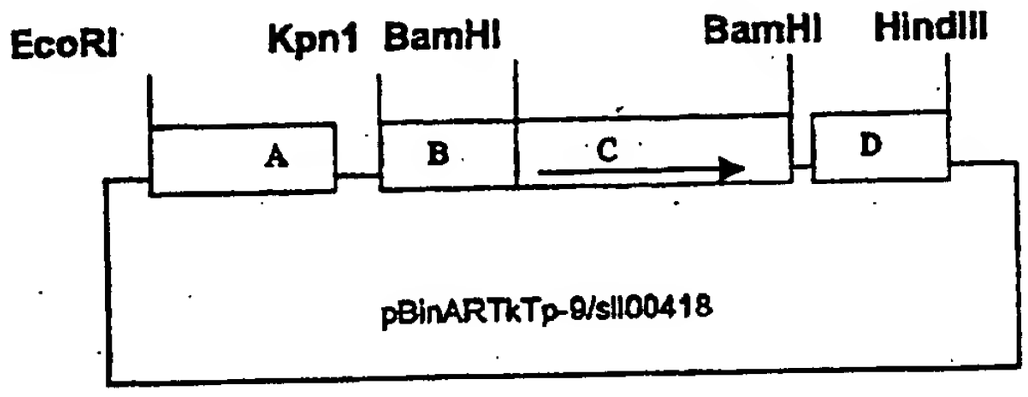
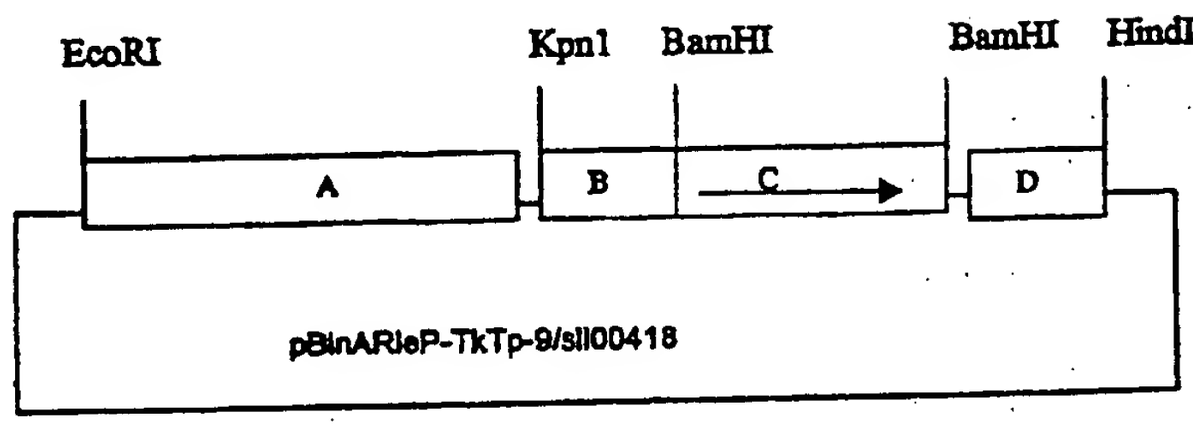


Abbildung 4



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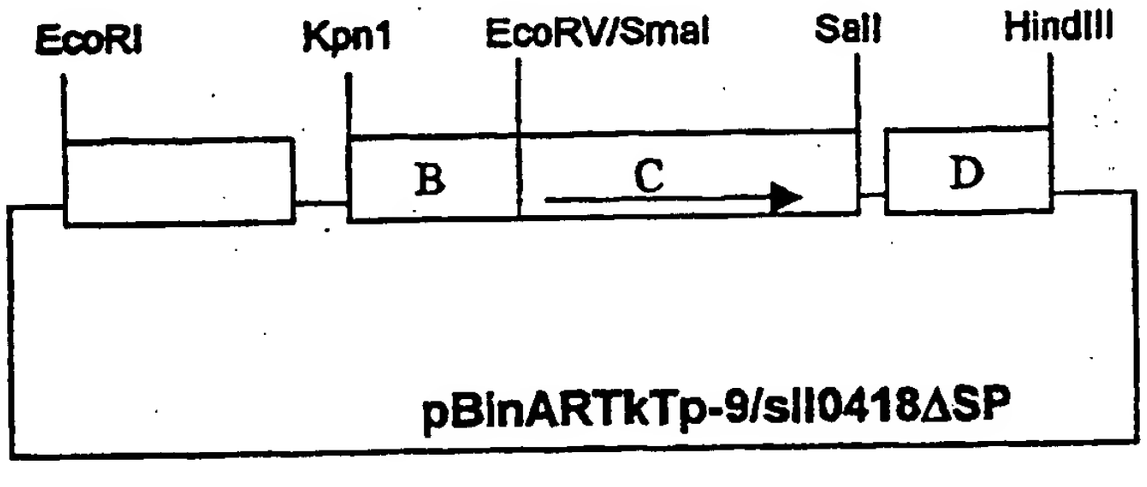

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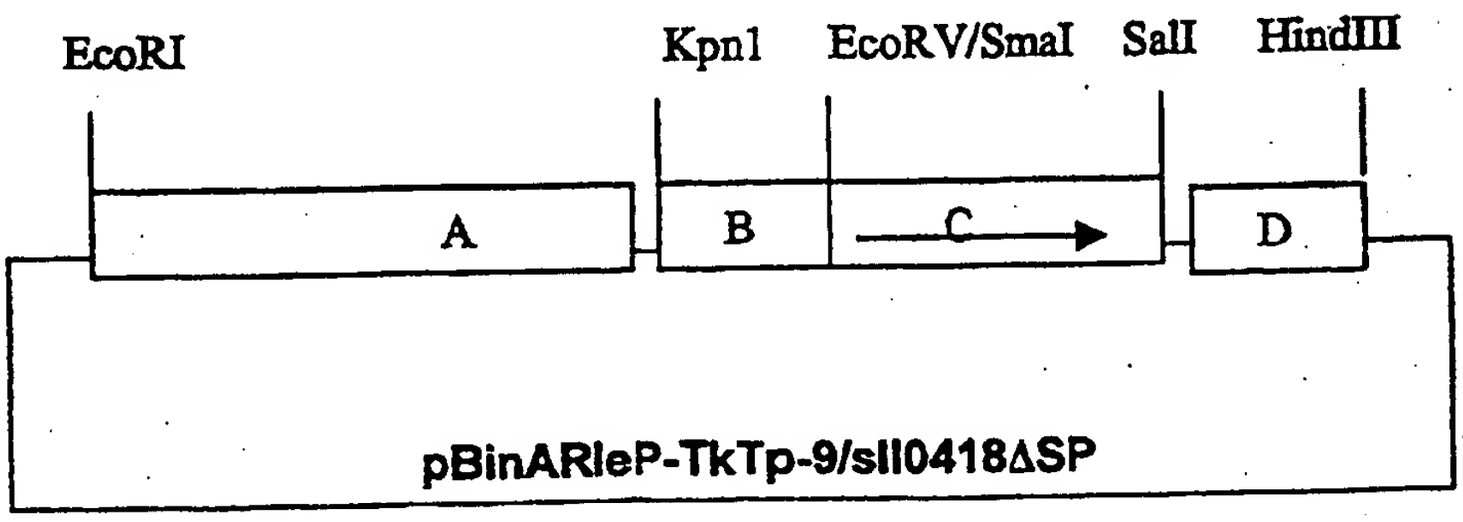

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Leu Glu Tyr Tyr Trp Gly Asp His Ile His Leu Gly His Tyr Gly Asp
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Pro Ala Trp Leu Asp Thr Ile Trp Gln Gly Ile Ile Arg Pro Gln Gly
260 265 270

tgg tta caa tac ggc att cgt ggg ttt atc aaa tcc gtg cgg gaa gta 864
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275 280 285

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Trp Glu Lys Pro Val Met Arg Gln Leu Leu Asp Gln Trp Ser His Pro
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Pro Ala Trp Leu Asp Thr Ile Trp Gln Gly Ile Ile Arg Pro Gln Gly
            260                 265                 270

Trp Leu Gln Tyr Gly Ile Arg Gly Phe Ile Lys Ser Val Arg Glu Val
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tcc ctg gcg atc gcc gct gga ctg tat ctc cta act gcc cgg ggc tat    96
Ser Leu Ala Ile Ala Ala Gly Leu Tyr Leu Leu Thr Ala Arg Gly Tyr
 15                  20                  25                  30

cag tca tcg gat tcc gtg gcc aac gcc tac gac caa tgg aca gag gac    144
Gln Ser Ser Asp Ser Val Ala Asn Ala Tyr Asp Gln Trp Thr Glu Asp
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ggc gat ccg cca gtg gcc aag gat ttc atc caa tcg aaa att gat ttt   240
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Val His Ala Met Ala Gln Trp Gly Gly Leu Asp Thr Leu Pro Pro Gly
    80                  85                  90

aca acg gta ttg gat gtg ggt tgc ggc att ggc ggt agc agt cgc att   336
Thr Thr Val Leu Asp Val Gly Cys Gly Ile Gly Gly Ser Ser Arg Ile
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ctc gcc aaa gat tat ggt ttt aac gtt acc ggc atc acc att agt ccc   384
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caa cag gtg aaa cgg gcg acg gaa tta act cct ccc gat gtg acg gcc   432
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cag ggc tgg tta caa tac ggc att cgt ggg ttt atc aaa tcc gtg cgg   864
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                275                 280                 285

gaa gta ccg act att tta ttg atg cgc ctt gcc ttt ggg gta gga ctt   912
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        35                  40                  45

Leu Glu Tyr Tyr Trp Gly Asp His Ile His Leu Gly His Tyr Gly Asp
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Pro Pro Val Ala Lys Asp Phe Ile Gln Ser Lys Ile Asp Phe Val His
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Ala Met Ala Gln Trp Gly Gly Leu Asp Thr Leu Pro Pro Gly Thr Thr
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165 170 175

Phe Ala Lys Glu Leu Leu Arg Val Val Lys Pro Gly Gly Ile Leu Val
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195 200 205

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210 215 220

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Trp Leu Gln Tyr Gly Ile Arg Gly Phe Ile Lys Ser Val Arg Glu Val
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att ttg gaa tat tac tgg ggc gac cat atc cac ctc ggc cat tat ggc 147
Ile Leu Glu Tyr Tyr Trp Gly Asp His Ile His Leu Gly His Tyr Gly
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    240                 245                 250

ggc tgg tta caa tac ggc att cgt ggg ttt atc aaa tcc gtg cgg gaa   819
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100 105 110

Lys Arg Ala Thr Glu Leu Thr Pro Pro Asp Val Thr Ala Lys Phe Ala
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195 200 205

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INTERNATIONAL SEARCH REPORT

Intern ıal Application No
PCT/EP 00/05862

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/82 C12N15/54 C12N9/10 C12N15/31 G01N33/53
A01H5/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N G01N A01H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, BIOSIS, PAJ, WPI Data, STRAND

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DATABASE EMBL SEQUENCE DATABASE 'Online! 31 October 1996 (1996-10-31) KANEKO, T., ET AL. : "sequence analysis of the genome of the unicellular cyanobacterium Syecchocystis sp. PCC6803. II. sequence determination of the entire genome and assignment of the potential protein-coding regions" XP002152668 accession no. D90914 --- -/--	1

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
22 November 2000	04/12/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	Holtorf, S

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INTERNATIONAL SEARCH REPORT

Interr ·ial Application No
PCT/EP 00/05862

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	HOEFGEN R ET AL: "BIOCHEMICAL AND GENETIC ANALYSIS OF DIFFERENT PATATIN ISOFORMS EXPRESSED IN VARIOUS ORGANS OF POTATO SOLANUM-TUBEROSUM" PLANT SCIENCE (LIMERICK), vol. 66, no. 2, 1990, pages 221-230, XP000964790 ISSN: 0168-9452 cited in the application page 223, left-hand column ---	5,6
A	WO 99 04622 A (UNIV NEVADA) 4 February 1999 (1999-02-04) cited in the application ---	
P,X	WO 00 10380 A (UNIV NEVADA) 2 March 2000 (2000-03-02) the whole document ---	1-8
P,X	WO 00 32757 A (RAFALSKI J ANTONI ;DU PONT (US); COUGHLAN SEAN J (US); MIAO GUO HU) 8 June 2000 (2000-06-08) the whole document -----	9,10

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Information on patent family members

Intern ıal Application No
PCT/EP 00/05862

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9904622 A	04-02-1999	AU 8506198 A	16-02-1999
		EP 1009812 A	21-06-2000
WO 0010380 A	02-03-2000	AU 5786199 A	14-03-2000
WO 0032757 A	08-06-2000	AU 2037700 A	19-06-2000

INTERNATIONALER RECHERCHENBERICHT

Intern ales Aktenzeichen
PCT/EP 00/05862

A. KLASSIFIZIERUNG DES ANMELDUNGSGEGENSTANDES
IPK 7 C12N15/82 C12N15/54 C12N9/10 C12N15/31 G01N33/53
A01H5/00

Nach der Internationalen Patentklassifikation (IPK) oder nach der nationalen Klassifikation und der IPK

B. RECHERCHIERTE GEBIETE

Recherchierter Mindestprüfstoff (Klassifikationssystem und Klassifikationssymbole)
IPK 7 C12N G01N A01H

Recherchierte aber nicht zum Mindestprüfstoff gehörende Veröffentlichungen, soweit diese unter die recherchierten Gebiete fallen

Während der internationalen Recherche konsultierte elektronische Datenbank (Name der Datenbank und evtl. verwendete Suchbegriffe)
EPO-Internal, BIOSIS, PAJ, WPI Data, STRAND

C. ALS WESENTLICH ANGESEHENE UNTERLAGEN

Kategorie°	Bezeichnung der Veröffentlichung, soweit erforderlich unter Angabe der in Betracht kommenden Teile	Betr. Anspruch Nr.
X	DATABASE EMBL SEQUENCE DATABASE 'Online! 31. Oktober 1996 (1996-10-31) KANEKO, T., ET AL. : "sequence analysis of the genome of the unicellular cyanobacterium Syecchocystis sp. PCC6803. II. sequence determination of the entire genome and assignment of the potential protein-coding regions" XP002152668 accession no. D90914 --- -/--	1

[X] Weitere Veröffentlichungen sind der Fortsetzung von Feld C zu entnehmen

[X] Siehe Anhang Patentfamilie

° Besondere Kategorien von angegebenen Veröffentlichungen :

"A" Veröffentlichung, die den allgemeinen Stand der Technik definiert, aber nicht als besonders bedeutsam anzusehen ist

"E" älteres Dokument, das jedoch erst am oder nach dem internationalen Anmeldedatum veröffentlicht worden ist

"L" Veröffentlichung, die geeignet ist, einen Prioritätsanspruch zweifelhaft erscheinen zu lassen, oder durch die das Veröffentlichungsdatum einer anderen im Recherchenbericht genannten Veröffentlichung belegt werden soll oder die aus einem anderen besonderen Grund angegeben ist (wie ausgeführt)

"O" Veröffentlichung, die sich auf eine mündliche Offenbarung, eine Benutzung, eine Ausstellung oder andere Maßnahmen bezieht

"P" Veröffentlichung, die vor dem internationalen Anmeldedatum, aber nach dem beanspruchten Prioritätsdatum veröffentlicht worden ist

"T" Spätere Veröffentlichung, die nach dem internationalen Anmeldedatum oder dem Prioritätsdatum veröffentlicht worden ist und mit der Anmeldung nicht kollidiert, sondern nur zum Verständnis des der Erfindung zugrundeliegenden Prinzips oder der ihr zugrundeliegenden Theorie angegeben ist

"X" Veröffentlichung von besonderer Bedeutung; die beanspruchte Erfindung kann allein aufgrund dieser Veröffentlichung nicht als neu oder auf erfinderischer Tätigkeit beruhend betrachtet werden

"Y" Veröffentlichung von besonderer Bedeutung; die beanspruchte Erfindung kann nicht als auf erfinderischer Tätigkeit beruhend betrachtet werden, wenn die Veröffentlichung mit einer oder mehreren anderen Veröffentlichungen dieser Kategorie in Verbindung gebracht wird und diese Verbindung für einen Fachmann naheliegend ist

"&" Veröffentlichung, die Mitglied derselben Patentfamilie ist

Datum des Abschlusses der internationalen Recherche	Absendedatum des internationalen Recherchenberichts
22. November 2000	04/12/2000
Name und Postanschrift der Internationalen Recherchenbehörde Europäisches Patentamt, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Bevollmächtigter Bediensteter Holtorf, S

2

Formblatt PCT/ISA/210 (Blatt 2) (Juli 1992)

INTERNATIONALER RECHERCHENBERICHT

Intern. ales Aktenzeichen
PCT/EP 00/05862

C.(Fortsetzung) ALS WESENTLICH ANGESEHENE UNTERLAGEN

Kategorie°	Bezeichnung der Veröffentlichung, soweit erforderlich unter Angabe der in Betracht kommenden Teile	Betr. Anspruch Nr.
X	HOEFGEN R ET AL: "BIOCHEMICAL AND GENETIC ANALYSIS OF DIFFERENT PATATIN ISOFORMS EXPRESSED IN VARIOUS ORGANS OF POTATO SOLANUM-TUBEROSUM" PLANT SCIENCE (LIMERICK), Bd. 66, Nr. 2, 1990, Seiten 221-230, XP000964790 ISSN: 0168-9452 in der Anmeldung erwähnt Seite 223, linke Spalte ---	5,6
A	WO 99 04622 A (UNIV NEVADA) 4. Februar 1999 (1999-02-04) in der Anmeldung erwähnt ---	
P,X	WO 00 10380 A (UNIV NEVADA) 2. März 2000 (2000-03-02) das ganze Dokument ---	1-8
P,X	WO 00 32757 A (RAFALSKI J ANTONI ;DU PONT (US); COUGHLAN SEAN J (US); MIAO GUO HU) 8. Juni 2000 (2000-06-08) das ganze Dokument -----	9,10

INTERNATIONALER RECHERCHENBERICHT

Angaben zu Veröffentlichungen, die zur selben Patentfamilie gehören

Intern. ales Aktenzeichen
PCT/EP 00/05862

Im Recherchenbericht angeführtes Patentdokument	Datum der Veröffentlichung	Mitglied(er) der Patentfamilie	Datum der Veröffentlichung
WO 9904622 A	04-02-1999	AU 8506198 A	16-02-1999
		EP 1009812 A	21-06-2000
WO 0010380 A	02-03-2000	AU 5786199 A	14-03-2000
WO 0032757 A	08-06-2000	AU 2037700 A	19-06-2000

SEQUENCE OF THE AMPLIFIED 209 bp PCR–PRODUCT OF THE β–GLOBIN GENE

FORWARD PRIMER: β2
CATTTGCTTC TGACACAACT GTGTTCACTA GCAACCTCAA ACAGACACCA
12mer PRIMER
TGGTGCACCT GACTCCTGTG GAGAAGTCTG CCGTTACTGC CCTGTGGGGC
AAGGTGAACG TGGATGAAGT TGGTGGTGAG GCCCTGGGCA GGTTGGTATC
AAGGTTACAA GACAGGTTTA AGGAGACCAA TAGAAACTGG GCATGTGGAG

ACAGAGAAG
REVERSE PRIMER β11

FIG. 51